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National Institutes of Arthritis, ... Digestive Diseases.
Report of program activities.

NIAMDD

ANNUAL PROJECT REPORTS

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NIAMDD ANNUAL REPORT

Fiscal Year 1979

OFFICE OF THE DIRECTOR

The programs of the National Institute of Arthritis, Metabolism, and Digestive Diseases are of profound importance to the American people, dealing as they do with a wide range of categorical disease responsibilities. The magnitude and severity of health problems presented by these diseases is extreme, and the urgency of the efforts necessary to reduce their toll of premature death, disability, and human suffering is immense. These diseases include arthritis and connective tissue diseases, skin diseases such as psoriasis; diseases of the bone and musculoskeletal system; diabetes, cystic fibrosis and other metabolic and endocrine disorders; digestive diseases and nutritional disorders; diseases of the kidney and urinary tract; and blood diseases.

Institute responsibilities in many of these areas have expanded greatly as a result of special legislation concerning arthritis, diabetes, and digestive diseases. This legislation has resulted in ambitious plans of action, which have begun to be implemented in recent years. The Institute is responsible for conducting, supporting and fostering research on diseases that affect all ages and sexes, all ethnic groups and strata of society, research of fundamental importance to virtually every citizen in our country.

More than 31 million people in the U.S., for instance, are afflicted with one or more of the 100 types of arthritis and other rheumatic and connective tissue diseases, at a cost to the nation of over $14 billion annually. The national arthritis program conducted and supported by NIAMDD is continuing investigations of abnormal connective tissue activation in rheumatic diseases, the joint-eroding effects of collagenase and lysosomal enzyme release and of immune complexes in rheumatoid arthritis, immunologic derangements in systemic lupus erythematosus (SLE), the mechanics of normal and arthritic joint action and of prostheses (artificial joints), and many other related subjects.

Both the National Arthritis Act (P.L. 93-640) and the recommendations of the National Commission on Arthritis and Related Musculoskeletal Diseases called for intensified action to apply currently known treatments for arthritis, to teach necessary professional skills, to demonstrate and stimulate prompt and effective application of available knowledge for treatment of patients, and to develop new knowledge through Multipurpose Arthritis Centers. The NIAMDD Centers Program has grown to a current level of twenty-four Centers in eighteen states, from New Hampshire to Hawaii, and additional applications are being reviewed. The Second Annual Center Director's Meeting was held on the NIH campus in March 1979, and representatives presented summaries

of the ongoing activities and planned cooperative endeavors among the various Centers.

The Institute also supports a broad program of research in orthopedics and bone diseases, including basic and clinical studies of skeletal tissues under normal and disease conditions. There are projects on calcium metabolism and bone formation, fracture healing and fixations, bone transplantation and preservation of skeletal tissue, prostheses (such as artificial joints) and relevant biomaterials, biomechanics and bioelectric phenomena, musculoskeletal dynamics and physiology of normal and abnormal gait. The areas of osteoporosis and osteogenesis imperfecta are receiving special program emphasis. The metabolism of cartilage is being studied at various centers throughout the country. A portion of funding in the fracture area has been used to investigate improved bone healing through new methods of electric stimulation.

Studies to determine research needs and priorities in the field of dermatology have been conducted and the final report of this analysis, performed under contract at the University of Washington, is in final preparation. The Coordinating Committee of this major effort is also preparing summary statements including the appraisal of current manpower resources, needs for additional research personnel, and an appraisal of technological development in this field. The Committee will submit a plan setting forth priorities for research, which will be published in the Journal of Investigative Dermatology.

Enactment in 1974 of the National Diabetes Mellitus Research and Education Act has led to an unprecedented research attack on diabetes by NIAMDD, as the lead agency for other NIH components and Federal agencies. Since this new initiative began, the Institute has strengthened existing programs and undertaken many new ones. Eight Diabetes Research and Training Centers have now been established. Each conducts research into the causes, diagnosis and management of diabetes and its complications, and trains professionals for diabetes research and management of diabetic patients. The Centers also serve as focal points for effective transmission of research advances to health professionals and for the translation of these advances into improved care for patients. In addition, the NIAMDD supports two Diabetes-Endocrinology Research Centers, which conduct ongoing biomedical research. Research areas of special interest recently have been islet cell transplantation, insulin delivery systems, and epidemiology.

Diabetes is one of a broad spectrum of metabolic diseases and inborn errors of metabolism investigated through NIAMDD support. Cystic fibrosis (CF), a lethal inherited metabolic disorder, affects one infant in every 2,000 live births and costs the nation an estimated $200 million annually in treatment alone. The Endocrinology, Metabolic Diseases and Resources Programs Branch, established this year, serves as the focus for NIAMDD activities related to CF. The Institute has directed a major effort during fiscal year 1979 toward increasing awareness among investigators of the needs and opportunities in CF research. This initiative resulted from a series of recommendations in the report entitled: Cystic Fibrosis: State of the Art and Directions for Future Research Efforts. This valuable, comprehensive document, prepared through joint sponsorship with the National Heart, Lung and Blood Institute, involved the cooperation of a broad spectrum of scientists in relevant disciplines and

was completed by the Cystic Fibrosis Foundation (acting under a contract from both Institutes) in fiscal year 1978.

Digestive diseases hospitalize more Americans than any other group of disorders. They affect more than 18 million people and their economic cost is estimated at $16.5 billion a year or about $80 annually for every American. The NIAMDD digestive diseases program encompasses the full spectrum of research of organ systems associated with the gastrointestinal tract, including salivary glands, esophagus, stomach, pancreas, liver, gallbladder, and small and large intestines. Research activity is concerned with basic aspects of anatomy, histology, physiology, endocrinology, and biochemistry of the digestive tract in health and in disease states. Future efforts will be directed toward implementing the long-range plan recommended by the National Commission on Digestive Diseases. The Commission dealt with a wide range of problems, including incidence and duration of digestive diseases, the mortality rates resulting from such illness and the social and economic impact of these disorders. Congress is currently considering legislation to carry out three of the major recommendations of the Commission.

New implications for health from the field of nutrition are emerging rapidly. Scientists now believe that in six of the 10 leading causes of death from disease in the U.S. today -- heart disease, stroke, cancer, diabetes, atherosclerosis and cirrhosis of the liver -- diet may play a significant contributory role. The Nutrition Program of the Institute has continued to stress these priorities: study of human nutritional requirements and factors that influence these requirements; use of new technologies in the study of the function of nutrients; studies on iron nutrition, metabolism and deficiency; requirements and metabolic roles of trace minerals; basic studies on dietary fiber; research on the interaction of nutrition, immune competence, and infection; increased support for obesity research with the aim of prevention; and studies on supportive nutrition for hospitalized patients through Clinical Nutrition Research Units. Since 1965, the Malnutrition Panel of the U.S.-Japan Cooperative Medical Science Program has served as the primary vehicle through which international nutrition research activities are identified for support. The U.S. Malnutrition Panel has continued to support research relating to the nutritional health problems of Southeast Asian people; many of these are of universal scope and of importance to other populations including that of the United States. The objectives of this program as they are now defined are to study: (1) the influence of environmental and host factors on nutritional requirements; (2) health significance and methods of preventing iron deficiency; (3) interaction of nutrition, immune competence and infection; (4) the effects of nutrition on physical and mental development, behavior, physical capability and work performance; and (5) the health consequences of different dietary patterns and food intake.

NIAMDD supported or cosponsored a number of workshops and conferences in the area of nutrition during the past year, including the International Conference on "Effects of Alterations of Dietary Patterns and Food Habits on Health with Special Reference to Critical Analysis of Comparative Epidemiological Studies of Different Populations" in Osaka, Japan, and the workshop on "Nutritional Support of Patients: Research Directions for the 1980s" in Washington, D.C. In December 1978, about 200 experts convened under sponsorship by the Institute to discuss the current status of "Surgical Treatment of Morbid Obesity" in a

concensus conference held in an effort to establish agreement among knowledgeable experts about this drastic approach to clinical control of obesity.

The kidney and urologic diseases program represents a significant portion of the overall NIAMDD research effort. The many patients with chronic uremia and end-stage kidney disease represent but one facet of these diverse disorders. Investigators supported by NIAMDD are seeking new methods of preventive therapy, earlier diagnosis and more effective treatment through expanded knowledge and understanding of the basic mechanisms and causes of a host of disorders. Areas of investigation include study of glomerulonephritis, nephrosis, interstitial nephritis, kidney and bladder stones, polycystic kidney disease, diabetic nephropathy, analgesic-associated and other toxic nephropathies, immune disorders of the kidney, benign prostatic hyperplasia, and numerous other diseases.

During fiscal year 1979, the activities of the former Artificial Kidney/Chronic Uremia Program were integrated into the overall Kidney Disease and Urology Program. As a result of this administrative reorganization, three sub-programs were designated in the Institute's extramural program related to the urinary tract: the Renal Physiology and Pathophysiology Program, the Urology (including urolithiasis) Program, and the Chronic Renal Disease Program. In addition to these, the Hematology Program focuses research on the fundamental structure, function and metabolism of blood components. An evaluation of this program in relation to research needs was undertaken this year in cooperation with other Institutes of the NIH with blood programs, and a final report of this significant study should be available by 1981.

During the past year, the Kidney, Urologic and Blood Diseases Program provided partial support for numerous major international meetings, including the Joint U.S.-U.S.S.R. Symposium on Biological Membranes in Kiev, U.S.S.R., and the 1979 International Prostaglandin Conference in Washington, D.C. In addition, a Conference on Immune Mechanisms in Renal Disease, the first inter-disciplinary meeting of its kind ever held, was sponsored in collaboration with the National Institute of Allergy and Infectious Diseases at the NIH.

The staff of NIAMDD's Kidney, Urologic and Blood Diseases Program also cooperated with the Fogarty International Center of the NIH to produce the new monograph Prevention of Kidney and Urinary Tract Diseases. This volume along with the recent report on Research Needs in Nephrology and Urology will serve as a valuable guide in formation of a strategy for the most effective use of limited resources in reducing the morbidity and mortality of kidney and urinary tract diseases in the United States.

During this year, the Institute has continued its commitment to providing training for its personnel. Short-term training opportunities, for example, have been arranged for more than 94 secretarial-clerical employees, 12 supervisory-managerial personnel, and 179 members of the professional staff. The NIAMDD Equal Employment Opportunity Subcommittee on Upward Mobility, Development and Training has conducted workshops, seminars and training classes for over 100 employees over the past five years, and currently has 45 Institute employees in training. Activities currently offered include courses in basic chemistry, basic mathematics, and three new courses in tissue culture that were developed last year.

The Institute continues to conduct an active Equal Employment Opportunity Program. The NIAMDD EEO Advisory Committee meets monthly to discuss issues and concerns and to formulate recommendations to meet EEO objectives as spelled out in the Institute's Affirmative Action Plan. This year the Institute entered into an intra-agency agreement for the support of the Minority Biomedical Support Program with the Division of Research Resources and a cooperative agreement for support of Minority Access to Research Careers Program with the National Institute of General Medical Sciences. The intent of these programs is to increase the number of minority scientists engaged in biological research/ health sciences. Dr. John Abrell was appointed to coordinate and monitor the Institute's responsibility in the contracts compliance process.

The institutional initiatives described in this report, combined with basic and applied research efforts are expected to yield great benefits in disease prevention, lives saved, and in extension of human life of maximal quality and productivity.

Membrane Regulation Section

Mechanism of Action of Hormones and Guanine Nucleotides on Adenylate Cyclase Systems

We have continued the use of high energy irradiation analysis for determining the size and composition of adenylate cyclase systems under various "ground-state" and ligand (hormones, guanine nucleotides, and fluoride)-induced states of the enzyme. Last year we reported that the glucagon-sensitive system in liver membranes consists of a multimeric form of the regulatory components (receptor and nucleotide regulatory components) which from binding studies suggests that the complex is a multimer of receptor (R) and nucleotide (N) binding proteins. We have speculated that this complex $(RN)^n$ breaks down to monomeric units (RN) which then form the holoenzyme (RNC). During the past year we have found that the activation of the fat cell adenylate cyclase system by various hormones (epinephrine, ACTH, and glucagon) in the presence of GTP shows the identical size patterns observed with the hepatic enzyme. In addition, we have evaluated the size of the GTP-inhibitory process that we think is a separate molecular process from the GTP-activating process. Indeed, by target analysis, it would appear that the GTP-inhibitory process is considerably larger in size than the GTP-activating process. Thus, target analysis has provided another means of probing for different structures controlling the complex adenylate cyclase system.

More recently, we have identified the catecholamine-sensitive adenylate cyclase system in turkey erythrocytes as having properties very different from liver and fat cell adenylate cyclase. This system is activated by hormone plus Gpp(NH)p but not by hormone plus GTP, the naturally occurring guanine nucleotide. It also binds GDP very tightly at the N unit such that hormone binding to R is weak. After pretreatment with hormone plus a non-activating nucleotide (GMP), GDP is released and the receptor acquires a tight-binding configuration; as with the liver system, this tight-binding configuration is promoted to a weaker binding state by addition of GTP or GDP. From various kinetic evaluations of the system it would appear that the receptor is pre-coupled to the enzyme system rather than being segregated in the large aggregate $(RN)^n$ regulatory complex seen in liver and fat cells. Based on target analysis this kinetic data has taken new significance since the size of the ground state enzyme is the same as that of the activated enzyme (RNC) in the presence of hormone and Gpp(NH)p (or GTP). Thus, the turkey erythrocyte adenylate cyclase system seems to have acquired an unusual configuration, possibly as the result of removal of some important controlling constituent during purification of the membranes. Interestingly, cholera toxin treatment of the membranes in the presence of its substrate NAD results in restoration of the turkey to the large ground-state regulatory form of the enzyme observed with liver adenylate cyclase after such treatment. We are continuing studies on the turkey system using high energy irradiation analysis and other probes to determine what the "normal" control factors are that seem to be responsible for maintaining the regulatory components

in their multimeric state. This may be decisive in understanding how adenylate cyclase systems in general respond to extraordinarily low concentrations of hormones with the marked amplification in response usually seen in the living cell. (W. Schlegel, P. Lad, T. Nielsen, M. Rodbell)

Purification of the Guanine Nucleotide Regulatory Component (N)

We began this year an intensive, collaborative study with Dr. Aurbach's laboratory to purify the nucleotide regulatory component (N) from turkey erythrocyte and possibly other sources of this essential component in adenylate cyclase regulation. Using a GTP-Sepharose affinity column, it is possible to concentrate the unit after detergent solubilization of turkey erythrocyte membranes. For assay of the N unit we are using a "complementation" assay which involves addition of N to membranes isolated from an SV49 lymphoma cell variant (termed N^-) that contains a MnATP form of adenylate cyclase and beta adrenergic receptors. These membranes lack the N units and do not respond to hormones, guanine nucleotides, cholera toxin, or fluoride ion. When N units are added, the system acquires a MgATP dependent activity and responds to the above regulatory agents. The value of this assay has been shown also for screening various membranes for their content of N units. Human erythrocyte (HE) membranes were found to contain concentrations of N unit comparable to those found in many other membranes. However, HE is very low in cyclase and receptor content. These findings allowed us to determine the properties of N when it is not linked to R or C in membranes. Thus, using complementation analysis with lymphoma N^- membranes and membranes (no detergent added) from HE it was found that free N poorly binds guanine nucleotides whereas when the NC unit is formed, guanine nucleotides bind tightly. The N unit also contains sulfhydryl groups that are important for the formation of NC. Finally, it was found that the HE N unit is responsible for activation of adenylate cyclase by guanine nucleotides, fluoride, and cholera toxin. We are investigating whether the same unit is responsible for activation by hormones. (P. Lad, T. Nielsen)

Actions of Adenosine

We established previously that plasma membranes of animal cells contain adenosine receptors (termed R-site) that are located on the outer surface of the membrane where most hormone receptors are located. In most cases studied thus far this class of receptors is linked to an N unit that mediates activation of adenylate cyclase. However, the R-type receptor in fat cells is linked to an inhibitory N process; this type of R-inhibitory process is physiologically relevant to the marked inhibition of cyclic AMP and lipolysis observed in isolated adipocytes. We have completed pharmacological studies that further classify adenosine receptors. Using deoxy-ATP as substrate (in order to avoid production of adenosine seen with ATP as substrate) it was possible to show that R-site activating and R-site inhibitory receptors have different specificities. Thus, in liver and Leydig tumor cell cyclase systems the R site (stimulatory) exhibits, in decreasing order of potency, the series: NECA (5'-ethylcarboxamideadenosine) > adenosine > PIA(N^6-phenylisopropyladenosine) whereas the R-site (inhibitory in fat cell) exhibits: PIA > adenosin > NECA. This classification holds true for the physiological responses of these agents in intact cells. Thus R-site adenosine receptors have, by analogy to alpha and beta adrenergic receptors, differing receptors

for inhibition and stimulation. In addition to alpha-R and beta-R type receptors, a third adenosine receptor, termed P-site, is ubiquitously linked to adenylate cyclase systems and confers only inhibition. We are intensively studying the P-site receptors since it may be a separate component from R, N, and C; we think it could be essential to the regulation of the enzyme system by divalent cations and hormones. (C Londos, D. Cooper, in collaboration with J. Wolff)

Relationship Between Membrane Regulation and Cellular Differentiation

As a means of understanding the possible relationship between cell membrane-mediated regulatory processes and the process of differentiation, we have studied the appearance and loss of beta adrenergic- and glucagon stimulation of the adenylate cyclase system in cell cultures. Loss of beta adrenergic response frequently occurs during cell culture. We had considered secretion of proteases as one possible cause of such losses since protease release often accompanies cellular transformation. However, addition of protease inhibitors failed to diminish the loss of receptors during culture.

A group of chemicals having the trait of inducing differentiation of cultured cells share the ability of inducing beta adrenergic response in several cell types. Using specific labeled beta-adrenergic binding agents (^{125}I-hydroxybenzylpindolol, ^{3}H-alprenalol), it was found that enhanced receptor input during cellular differentiation accompanied increased hormone responsiveness of adenylate cyclase. Treatment with sodium butyrate, a notable differentiating agent, resulted in enhanced catecholamine response in HeLa cells; induction was not dependent on the cell cycle since little variation occurred after cells were synchronized with thymidine blockade. A decrease in stimulation by catecholamines was observed during the mitotic phase. In collaboration with Dr. Lois Tice, we have found that butyrate treatment results in a dramatic increase in vesicle formation and the differentiation-inducing properties of butyrate and other chemical differentiating agents is being explored.

Finally, we have been successful in establishing an adult rat hepatocyte culture which retains its responsiveness to hormones (epinephrine, glucagon, and prostaglandins) after several weeks in culture. In addition to serum, insulin and hydrocortisone are required for maintenance of the adult cell culture. We are continuing studies with a fetal hepatocyte cultured line and with a temperature-sensitive SV40 transformed fetal cell line which look promising for relating cell growth and differentiation and the processes involved in the regulation of receptor function and concentration in the plasma membrane. (M. Lin)

Structure-function Relationships in Glucagon

We are mapping the regions in glucagon that are responsible for recognition, activation, and high potency of the molecule seen with the glucagon-sensitive cyclase system in liver membranes. A major advance was the finding that glucagon$_{1-6}$ carries out activation of adenylate cyclase and binds to the glucagon receptor. As expected, its binding affinity is greatly weakened relative to that of native hormone. Nonetheless, this finding indicates that

action only requires the first six residues of the hormone. Since the first seven residues of vasointestinal peptide (VIP) and secretin are homologous with those of glucagon, we have synthesized VIP_{1-7} and $secretin_{1-7}$. Neither interacts with the glucagon receptor. Since their major difference from glucagon is replacement of $glutamine_3$ with aspartate it would appear that recognition and action of $glucagon_{1-6}$ depends on glutamine. $Glucagon_{1-12}$ fully stimulates adenylate cyclase with slightly increased potency over $glucagon_{1-6}$. As expected, $glucagon_{13-29}$ failed to activate adenylate cyclase. However, it binds to the receptor since it displaces native glucagon with 0.01 the affinity of the latter. It would appear therefore that the glucagon receptor has separate domains for binding the active region ($glucagon_{1-6}$) and for tight binding (regions proximal to the C-terminal portion).

We have completed studies on the modification of the lysyl and tryptophan residues through amidination and attachment of agents to modified SH-tryptophan. All analogues of these residues give high potencies and are fully active. We plan to substitute labeled photo-reactive groups in order to tag the glucagon receptor for isolation and purification. (D. Wright)

The GTP-Inhibitory Process

We had previously shown that the fat cell contains a GTP-dependent inhibitory process that mediates the inhibitory effects of adenosine on cyclase activity and lipolysis in fat cells. As a means of further exploring GTP-inhibitory processes in other types of cells we have turned to the human platelet. PGE_1 stimulates cyclic AMP production whereas catecholamines (through an alpha adrenergic receptor) inhibit production. We have found that the former is dependent on GTP.

We now have established that GTP is also required for the inhibitory effects of catecholamines (norepinephrine). In addition, we have discovered recently that ADP, acting through a distinct purinergic receptor, inhibits adenylate cyclase in platelets; the K_i is about 0.5 μM which approximates the concentration at which ADP promotes platelet aggregation. We think, therefore, that the effects seen with ADP on cyclic AMP represent the physiological actions of this nucleotide. We are in the process of determining whether this action of ADP is linked to the GTP-inhibitory process that mediates the actions of alpha adrenergic compounds on platelets. (D. Cooper)

Developmental Biochemistry Section

Chromatin Assembly

Others have reported that assembly of SV-40 minichromosomes from histones and DNA at near physiologic ionic strength requires the participation of "assembly factors." In contrast, we find that in the absence of any extraneous factors, at proper protein:DNA ratios, a partial concerted assembly occurs over the course of 16 hrs at 37°C. A marked acceleration of the rate of assembly and enhancement of the degree of correct reassociation eventually attained is achieved by adding polyglutamic acid to the reaction mixture. Thus, incubation of SV-40 DNA[I] with a 2:1 ratio of histones and 4:1 of polyglutamic acid for 60 min at 37° leads to nearly quantitative conversion

of the DNA to a superhelical form after relaxation and deproteinization. Other experiments, using crosslinking, demonstrate that histones are associated as octamers at I = 0.1-0.2 when bound to polyglutamic acid; this contrasts with their dissociation into dimers and monomers in the absence of the polyanion. The acidic protein thus maintains the histones in the state required for their interaction with DNA. Since they facilitate assembly, acidic proteins might also facilitate disassembly of chromatin, an attractive mechanism for explaining previously observed stimulatory effects of acidic proteins on transcription. (A. Stein)

Core Particles Constructed With Simple Sequence DNA

Resolution in studies of histone-DNA interactions in core particles has been limited by the sequence heterogeniety of DNA in native particles; this affects both physical and biochemical studies. To surmount this problem we have constructed semisynthetic core particles from histones and the alternating copolymers, poly(dAdT)·poly(dAdT) and poly(dGdC)·poly(dGdC). The core particles are highly homogeneous, containing 145±1 base pairs of DNA. DNAase I digestion and cutting site mapping to single base resolution in the AT particles shows that every site can be cut by this nuclease, not only sites at multiples of about 10; this observation rules out static kinking as the path for DNA in the nucleosome. The exact repeat length between preferential cutting sites was determined to be 10.4 base pairs. A number of other features of core particle structure are much better defined, or even revealed, in these particles compared to native particles with random sequence DNA.

Using the AT particles we have been able to accurately describe the mechanism of a reversible conformational change induced by mild heating of the particles. At temperatures 10° below optical indications of strand separation for 30% of the DNA, the particle shape changes to become more elongated and about 30% of the DNA is freed from tight binding to the histone octamer, indicated by circular dichroism and ^{31}P-NMR measurements. S1 nuclease digestion of partially melted, 5 -end labeled particles shows clearly that the first-melted DNA is at the ends of the core particle segment. Thus, the core particle can unwind from the ends, leaving a structure which has barely one full turn of DNA around the histone octamer. This is a plausible intermediate in opening up chromatin structure during transcription.

^{31}P-NMR studies of the AT particles have 1) precluded kinking of DNA in nucleosomes, 2) suggested heterogeneity in the conformation of the phosphodiester backbone and/or its interactions with histones along the length of core particle DNA, and 3) shown for the first time a sequence dependent variation in the geometry of the phosphodiester backbone of double helical DNA. (R. Simpson, P. Kunzler, H. Shindo)

Histone Modification and Higher Order Chromatin Structure

Hyperphosphorylated H1 histone-containing nuclei were compared to HTC cell nuclei with hypophosphorylated H1. Indications of a looser organization

for higher levels of chromatin structure were obtained for the cells with hyperphosphorylated histone. Thus, for the phosphorylated samples, micrococcal nuclease excision of superbeads (about 8 nucleosomes) was more rapid, the sedimentation coefficient of chromatin of given DNA length was less, the chromatin did not further expand at low ionic strength, and the melting temperature was lowered by about 2°.

Using both unmodified and in vivo acetylated histones, we have examined the possibility of localized phasing of nucleosomes on SV-40 DNA. When reassociation is performed with either superhelical or relaxed DNA at a variety of temperatures, using salt gradient dialysis, a random distribution of nucleosomes is always observed upon analysis with single-cut restriction endonucleases. Thus, the phasing near the origin in native SV-40 must arise from interactions other than those between the DNA and the inner histones. (R. Simpson, M. O. Olson)

Interaction of Chromatin With Triiodothyronine (T3)

Using our previously described facile assay for study of the binding of T3 to its chromatin-associated receptor, we have examined the neighborhood of the receptor in chromatin. Digestion with nucleases, followed by centrifugation, shows that the receptor is solubilized early as a 6S entity. On prolonged digestion, its size is reduced to 5S, suggesting association of the initially released receptor with DNA. At all times, the receptor complex is larger than the putatively free receptor released by salt extraction of chromatin, 3.5S. These findings are consistent with localization of the T3 receptor in segments of transcriptionally active chromatin in rodent liver, perhaps associated with unfolded nucleosomes such as are found for the ribosomal genes by others. As an additional method for study of the size of the T3 receptor in situ, we have begun radiation inactivation studies using target size analysis. Preliminary results give good agreement for size of solubilized receptor by this and sedimentation methods; the size of the receptor in chromatin appears to be much larger, again suggesting a complex structure for the functional thyroid hormone receptor in the nucleus. (D. Gruol)

In Vitro Transcription of Chromatin and Isolated Genes

We have made a commitment to developing systems which will allow study of the accurate in vitro transcription of both defined gene sequences as DNA and determination of the features necessary and sufficient for transcription of these sequences when present in chromatin. In beginning these studies, we have isolated RNA polymerase II from yeast to near physical homogeniety and studied its interactions with isolated chromatin core particles. The polymerase transcribes core particles at a rate half that of protein-free DNA of the same length; this is much higher than observed for intact chromatin and suggests the participation of compositional and structural features at higher levels of organization in repression of transcription. Between two and four polymerase molecules bind to either substrate, indicating that the formation of a complex of DNA with histones as a core particle has remarkably little effect on the ability of the nucleic acid to interact with other proteins. Relevant to the mechanism of

transcription of core particle-bound DNA by RNA polymerase, we have determined that covalently crosslinked core particles are transcribed as readily as native particles; this suggests that histone dissociation may not be required for RNA synthesis on chromatin, although we do not yet know the size of the RNA synthesized in these experiments. (G. Bitter)

Structure - Function Studies of Nucleases

We have devised methods which lead, in a single step, to purification to enzymatic purity, or, in two steps, to purification to physicochemical purity, of restriction endonucleases. An initial chromatography on Cibacron Blue F3GA covalently linked to a matrix allows purification of a number of restriction endonucleases sufficient for their use as reagents. A second chromatographic fractionation on heparin-agarose leads to complete purification of the enzyme Pal I. This should provide a method for total purification of these nucleases for study of their mechanism of catalysis and their use as models for sequence-specific protein nucleic acid interactions. (G. Rushizky, K. Baksi)

Structural studies on the extracellular ribonuclease of B. amyloliquefaciens have resumed; crystal studies using both native molecules and those modified with iodine at a single tyrosyl residue are in progress. Recently published sequences of several other pro- and eukaryotic ribonucleases, in conjunction with our sequence determined for the B. amyloliquefaciens enzyme, have allowed us to define extensive homologies between enzymes of bacterial and lower eukaryotic origins. These ribonucleases thus join a small family of proteins for which there are indications of common DNA sequence origins in the prokaryotes and the eukaryotes. (R. Hartley)

Nutritional Biochemistry Section

Studies have continued on the basal metabolism of the small intestine and the contribution the intestine makes to total plasma lipoproteins (Windmueller). In the rat, liver and intestine were found to be the only sources of the constituent apoproteins of plasma lipoproteins. Intestine produced 19% of the total apolipoprotein pool -- 50-60% of apolipoprotein A-I (apoA-I) and apoA-IV, 16% of apoB, <10% of apoC and <1% of apoE. The quantitative order of synthesis in the intact animal was apoE > apoB > apoA-I > apoC > apoA-IV. ApoA-I and apoA-IV are major constituents of plasma high density lipoproteins, and so intestine contributes importantly to this lipoprotein class. In other studies with in situ autoperfused jejunal segments from fed rats, plasma glutamine was identified as the major respiratory fuel after the lumen was emptied. Glutamine nitrogen was released into portal blood as ammonia and alanine, substrates for urea synthesis in liver, and as citrulline, a substrate for arginine synthesis in kidney. During absorption of a typical meal, intestinal metabolism of luminal glutamine, glutamate and aspartate accounted for 40% of the respiratory CO_2 produced. These studies reveal the importance of glutamate and aspartate in intestinal energy metabolism and the quantitatively significant role of small intestine in processing circulating glutamine, a major nitrogenous end-product from muscle and other tissues.

Nutrition studies have explored the possible contribution of retinol (vitamin A) to the formation of the fluorescent pigment, lipofuscin, which accumulates in vitamin E deficient tissues (Bieri). Using the rat uterus as model, it was found that the formation of the pigment in this tissue was unaffected by the animal's vitamin A status. The amount of pigment was similar whether rats were deprived of retinol for five months or fed high amounts of the vitamin. It was concluded that vitamin A cannot be a significant component of lipofuscin. Quantification of the pigment by determining the fluorescence of lipid extracts from affected uteri was found not to give a reliable estimate of the extent of lipofuscin deposition. In continuing studies of evaluating nutritional status, a rapid micro method, utilizing high pressure liquid chromatography, was developed for the simultaneous determination of vitamins A and E in blood plasma and red cells.

Vitamin Metabolism Section

Dihydrofolate reductase continues to be of considerable pharmacological, biochemical, and now, genetic, interest because this enzyme appears to be the site of action of the powerful chemotherapeutic drug, methotrexate. Although the enzymes of bacterial origin have received the most attention, the corresponding enzymes of animal origin are not only more relevant to the problems of cancer therapy these enzymes appear to be unique in many of their biochemical properties. For example, many of the animal enzymes have been reported to exhibit an increase in catalytic activity upon treatment with organic mercurials whereas the corresponding bacterial dihydrofolate reductases are either unaffected or markedly inhibited by the same agents. Although the significance of this phenomenon is obscure it is tempting to speculate that all animal dihydrofolate reductases are activated by mercurials in contrast to the opposite or lack of effect on the bacterial enzymes and this, in turn, represents a fundamental difference between the catalytic properties of the two different sources of enzymes, animal and bacterial.

It would appear that the basis for these differences must reside in the nature of the sulfhydryl group or groups in the polypeptide chains of the particular dihydrofolate reductase protein. Therefore our current investigations have been concerned with the amino acid sequences of the chicken and bovine liver enzymes particularly the location of the cysteine residues. These studies have shown that each of the animal enzymes sequenced to date contain a single cysteine residue located in the amino terminal region of the protein: chicken liver, 11; bovine liver, 6; L1210 mouse lymphoma, 6. By contrast, none of the bacterial reductases sequenced to date contain a cysteine residue located within 25 amino acids of the amino terminus.

Since the function of this sulfhydryl group in the animal reductases is obscure we have continued to investigate its reactivity as well as the alteration of enzymatic activity and properties as a result of various sulfhydryl modifications. Thus, it has been recently found that when chicken liver dihydrofolate reductase reacts with an excess of sodium tetrathionate, one mole of thiosulfate becomes covalently attached to the single sulfhydryl group of the enzyme. The resulting sulfonyl thiosulfate form of the enzyme has 5- to 10-fold more activity than the native enzyme.

This is the first case of an enzyme exhibiting activation rather than inhibition upon treatment with this reagent. On the other hand, beef liver dihydrofolate reductase did not show any change in activity on treatment with the tetrathionate. (B. T. Kaufman and E. K. Barbehenn)

Endocrinology Section

Insulin Regulation of Lipoprotein Lipase Activity and Release in Fat Cells

In the previous year we established that the 3T3-L1 mouse fibroblast cell line, which differentiates into fat cells in culture, provides an excellent model system for examining hormonal regulation of lipoprotein lipase (LPL). We found that 1) LPL activity was extremely low in undifferentiated fibroblasts and increased ∿100 fold in fully differentiated fat cells, 2) biochemical properties of LPL in these cells were identical to that found in adipose tissue, 3) appearance of LPL was unaffected by the concentration of substrate, lipoprotein triacylglycerol, in the culture medium during differentiation, and 4) LPL activity in these cells could facilitate triacylglycerol deposition, independent of de novo fatty acid synthesis, during development of the preadipocyte. Since the appearance of LPL served as one of the earliest and most reliable markers of the differentiated state, we were able to describe the effects of a number of hormonal agents (insulin, prolactin, prostaglandin $F_{2\alpha}$, methylxanthine) on the developmental process. Insulin proved to be the most potent and important factor regulating development of LPL activity and development of triacylglycerol in these cells.

During the past year we have studied the mechanisms by which insulin regulates LPL in mature, fully differentiated 3T3-L1 fat cells. Low, physiologically relevant concentrations of insulin (10^{-10} to 10^{-7} M) exerted two effects on LPL in these cells, an increase in rate of release of the enzyme from cells to the medium (∿10 fold in 15 min), and a slower increase in total enzyme activity in the cells (3 to 10 fold in 2 days). Prolonged treatment with insulin also resulted in additional increases in enzyme release and a 10-fold increase in the fractional rate of release of enzyme per hour. Both effects mimic previously described actions of insulin on LPL activity in adipose tissue in vivo.

Stimulation of cellular lipoprotein lipase activity by insulin was dependent on the presence of glucose in the incubation medium. Although half-maximal increases in total activity required 12 hours of insulin treatment in glucose-containing medium, removal of either glucose or insulin resulted in loss within 90 min of >50% of the stimulation in enzyme activity. Addition of glucose to cells incubated 20 hours with insulin in carbohydrate-free medium elicited a rapid and full response to insulin in 2 hours. Fructose, mannose and glucosamine could substitute for glucose in supporting this rapid response in insulin-pretreated cells, but only glucose, mannose and fructose would support the effect in 24 hour-long incubations with insulin. Induction of increase in LPL by glucose in insulin-pretreated cells was prevented by cycloheximide, but not by actinomycin D. In contrast, inhibitors of either protein or RNA synthesis completely blocked the response to insulin in cells incubated 24 hours with glucose.

On the basis of these findings, we propose that insulin regulates lipoprotein lipase in fat cells through three major actions: 1) Insulin elicits rapid release of enzyme from cells through mechanisms which are independent of energy metabolism, protein synthesis, and functioning cytosketal elements. 2) Insulin stimulates gene expression and mRNA synthesis independently of any effect on hexose or amino acid transport. 3) Insulin stimulates expression of nuclear effects at the translational level by increasing hexose uptake and levels of regulatory glycolytic metabolites within the cells. Induction of the enzyme and its release thus appear part of a generalized pleotropic growth response mediated by separable effects on membrane function and macromolecular synthesis.

Studies are currently in progress to 1) identify hexose metabolites that regulate enzyme synthesis at the translational level and determine their mode of action, 2) determine the mechanism by which catecholamines and fatty acids inhibit LPL activity in these cells, and 3) produce specific antibodies to LPL isolated from 3T3L1 cells (P. M. Spooner, S. S. Chernick and R. O. Scow).

Insulin Receptors of Isolated Capillaries

The kinetics of insulin removal from peripheral circulation gave rise to the suggestion that the first step in insulin uptake by some tissues might be the binding of hormone to specific receptors on the luminal surface of capillary endothelium. In an effort to quantitate this phenomenon and to identify the physiological factors that affect insulin receptors on capillaries we have successfully prepared isolated capillaries and measured their binding of ^{125}I-insulin.

Capillaries were isolated from rat parametrial fat pads by digestion with collagenase, separation of stromo-vascular cells from fat cells by low speed centrifugation, and collection of capillaries (and other small blood vessels) on membranes or meshes of known pore size. Insulin binding and degradation were determined by procedures developed at NIAMDD. DNA was determined by a microfluorimetric procedure on the same sample used in the binding study.

The results indicate that isolated capillaries bind ^{125}I-insulin in a time-dependent manner. Specific binding reached a steady state at 23° C in 12 hours. The amount of insulin bound was a function of the DNA content of the sample. The amount of binding per unit DNA was greater in the larger vascular fractions (retained by 50 μm mesh) than in the smaller fractions (passed through 50 μm mesh but retained by 8 μm membrane). The free cell and cell fragment fraction (passed through 8 μm filter and collected by centrifugation at 200 g for 3 min) had the least capacity for binding insulin per unit DNA. Degradation of insulin by isolated capillaries was also time-dependent and about 10% of the added label was TCA-soluble in 1 hour.

Experiments in progress are designed to relate binding of insulin to its biological activity (increases glucose oxidation and lipogenesis) and to explore the relationship between the insulin receptor concentration of fat cells and that of capillaries in adipose tissue. Initial studies

suggest that fasting for 3 days may cause a doubling of insulin binding by isolated capillaries of adipose tissue.

Since the techniques for measuring insulin binding by isolated adipocytes and that by capillaries differ greatly, it is not advisable at present to compare the binding capacities of the preparations. Efforts are being made to relate the binding capacities of fat cells and capillaries from the same tissue in a variety of conditions and to develop a means for quantitative comparison (S. S. Chernick and R. O. Scow).

Lipid Transport Between Blood and Tissue by Lateral Movement in Cell Membranes

We proposed several years ago a model for transport of lipid from chylomicrons to tissue cells by lateral movement in cell membranes. The model requires 1) conversion of chylomicron triacylglycerol to amphipathic lipids, fatty acids and monoacylglycerol by lipoprotein lipase at the capillary surface, 2) location and lateral movement of these products in a continuous interface consisting of the chylomicron surface film and the external leaflets of plasma and intracellular membranes of endothelial and parenchymal cells, and 3) removal of these products from the interface in endoplasmic reticulum of cells where they are reesterified to triacylglycerol and separate from the interface, forming lipid droplets between leaflets of endoplasmic reticulum.

Morphological studies reported earlier of chylomicrons incubated with lipoprotein lipase and limited albumin showed that lipolytic products accumulated in the interface between triacylglycerol and the aqueous medium, extending the chylomicron surface film as a monolayer lining and spiraling within aqueous spaces that developed in the chylomicron core. Biochemical studies reported last year on effects of lipoprotein lipase on triacylglycerol at an argon-water interface, using a tricompartmented trough, showed that lipolytic products located and spread immediately throughout the interface. As they crowded the interface, surface pressure increased, lipolysis slowed down, and substances with lower spreading pressures were displaced from the interface. Addition of albumin to the aqueous subphase, to serve as a trapping agent, increased markedly desorption of fatty acids and monoacylglycerol from the interface, and thereby lowered surface pressure and enhanced lipolysis. Lateral movement of these lipids in the interface was demonstrated when lipoprotein lipase and albumin were added to separate compartments.

The concept that there is an interfacial continuum between chylomicrons and tissue cells during uptake of triacylglycerol from chylomicrons is supported by observations in rat tissues incubated after being fixed with glutaraldehyde. Biochemical studies showed that lipoprotein lipase is active in fixed tissue. Morphological studies of glutaraldehyde-fixed adipose tissue incubated at 38°C showed lamellar structures in vacuoles and vesicles of capillary endothelium and in spaces between endothelium and pericytes. These structures were not found in unincubated tissue. At the time of the study we concluded that the lamellar structures represented lipolytic products at sites of hydrolysis, and that hydrolysis occurred within vacuoles and vesicles of endothelial cells and in subendothelial

spaces. In view of our recent finding that fatty acids and monoacylglycerol can transfer from the site of hydrolysis by lateral movement in the interfacial plane between triacylglycerol and water, we now conclude that the lamellar structures represent primarily products, not sites, of hydrolysis. Since the main source of triacylglycerol for lipoprotein lipase activity in fixed tissue is chylomicrons attached to capillary endothelium, it seemed likely that in the above studies hydrolysis occurred at the surface of chylomicrons and lipolytic products moved across the capillary endothelium within the luminal leaflet of membranes of vesicles and vacuoles, and when their movement was impaired they formed lamellar structures within vesicles and vacuoles.

Lamellar structures were found also in mammary gland of lactating rats injected with chylomicrons, in capillary endothelium, in spaces between cells, and associated with cellular projections. When fixed mammary tissue was incubated at 38°C, lamellar structures were found near mammary secretory cells, indicating movement of lipolytic products during incubation. The continuity of plasma membrane with layers of lamellar structures suggested the lamellae might be formed as lateral extensions of membranes.

Heart derives much of its energy from fatty acids taken up from chylomicrons. Recent morphological studies of heart from adult rats injected with chylomicrons showed the presence of lamellar structures in capillary endothelium, in the spaces between endothelium and other cells, at the surface of myocytes, and associated with the tubular system and mitochondria of myocytes. Incubation of heart tissue after being fixed with glutaralydehyde increased the amount of lamellae seen at all sites. The lamellae associated with mitochondria could represent lipolytic products derived from fat stores in myocytes. This possibility is under study.

Adipocytes undergo rapid development and growth in epididymal and parametrial adipose tissue of young suckling rats. They readily take up fatty acids from chylomicrons during feeding, and release fatty acids from intracellular fat stores to the blood stream during fasting. Adipose tissue of 6-11 day old suckling rats was processed for electron microscopy with special fixative procedures developed to retain lipid in tissue. Morphological study of these tissues showed cytoplasmic processes of endothelial cells that extended to adipocytes and similar processes of adipocytes that extended to other cells, connections between the outer leaflet of plasma membrane of endothelial cells and that of fat cells, and continuity between plasma membranes and intracellular membranes in both endothelial and fat cells. These findings demonstrate the presence of a structural continuum between and within cells.

Many spiraling lamellae were found in sections of adipose tissue of suckling rats. The lamellae were continuous with membranes of both capillary endothelium and adipocytes. Experiments were designed to determine the relationship between distribution of lamellae and lipolytic activity in capillaries and fat cells. Epididymal and parametrial fat pads were taken from fed and fasted rats, and also from fed rats injected intravenously with chylomicrons. Lamellae were found primarily in and near capillary endothelium in adipose tissue from fed animals, and they increased in size

and number and also appeared near fat cells when the tissue was incubated at 24°C after being fixed with glutaraldehyde. The lamellae were considerably larger and more numerous in tissue from fed rats injected with chylomicrons. In tissue from fasted animals, lamellae were found primarily in and near fat cells, and they increased in size and number and appeared near capillaries when the tissue was incubated after being fixed. Lamellae were absent in adipose tissue of fed rats when the tissue was incubated with 5 mM glucose and 10^{-7} M insulin before being fixed. Biochemical studies of adipose tissue from suckling rats injected with chylomicrons containing triacyglycerol labeled with [^{3}H]oleic acid showed that chylomicron triacyglycerol was hydrolyzed to fatty acids when unfixed tissue was incubated in Tyrode's solution (pH 7.4) at 38°C, and that all of the fatty acids produced were immediately reesterified to triacylglycerol when glucose and insulin were added to the incubation medium. The absence of both lamellae and fatty acids in tissues incubated with glucose and insulin indicate that the lamellae are composed of lipolytic products.

We conclude that the lamellae in developing adipose tissue represent aggregations of lipolytic products within the interfacial continuum of membranes, and that they were formed during tissue preparation from chylomicrons in tissue from fed rats, and from fat stores in adipocytes of tissue from fasted rats. The changes in distribution of lamellae associated with incubation of fixed tissue, with lamellae first appearing near adipocytes in tissue from fed rats and near capillaries in tissue from fasted rats, demonstrate movement of lipolytic products from their respective sites of formation. Thus, the lamellae mark in fixed tissue the route, the membrane continuum, proposed for transit of lipolytic products from capillary lumen to parenchymal cells (E. J. Blanchette-Mackie, M. G. Wetzel and R. O. Scow).

Accumulation of Lipid in Muscular Arteries of Short-Term Diabetic Rats

Accumulation of lipid in muscular (pulmonary, coronary and tibial) and elastic (aorta and pulmonary) arteries of diabetic rats was studied with the electron microscope. Diabetes was induced with streptozotocin, and tissues were taken 4 days later. Lipid deposits were found in medial smooth muscle cells, near mitochondria and sarcoplasmic reticulum, in muscular arteries in 9 of 24 diabetic rats and in none of 17 normal control rats. Histochemical analyses demonstrated that the lipid in the cells was triacylglycerol. Lipid deposits were not seen in elastic arteries of either diabetic or normal rats. The diabetic rats had high plasma concentrations of glucose (320 mg/dl), free fatty acids (>0.77 mM), triacylglycerol (1,100 mg/dl) and cholesterol (127 mg/dl), and low plasma concentrations of insulin (<8 μU/ml). The primary differences in plasma between the two groups of diabetic rats were that plasma FFA concentrations were higher and insulin concentrations were lower in rats with arterial lipid deposits. The amount of lipid deposited in the arteries was closely related to plasma FFA concentration, which ranged 0.8-1.2 mM in diabetic rats without deposits and 1.1-2.5 mM in those with deposits. The findings suggest that lipid accumulation in smooth muscle cells of muscular arteries during acute diabetes results primarily from high concentrations of FFA in plasma (R. O. Scow with collaboration of A. Reinila and H. K. Akerblom of the University of Oulu, Finland).

Pilot Plant Unit

Production of large quantities of microorganisms for the isolation and purification of biologically important macromolecules and cell particulates and to assist NIH investigators in the scale-up and processing of plant and animal tissues for biological substances such as enzymes, vitamins, alkaloids, etc.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOI AM 15000-09 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mechanism of Action of Hormones and Guanine Nucleotides on Adenylate Cyclase Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Lad	Sr. Staff Fellow	LNE	NIAMDD
Others:	T.B. Nielsen	Staff Fellow	LNE	NIAMDD
	M. Sue Preston	Biologist	LNE	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The GTP-regulatory unit (N) of the turkey erythrocyte adenylate cyclase system contains tightly bound GDP which affects both the binding of isoproterenol to the receptor and prevents activation by the normal activator, GTP. Removal of bound GDP results in restoration of the enzyme system to a tighter binding configuration of the beta adrenergic receptor and to negative heterotropic effects of hormone and GTP on the receptor-nucleotide regulatory protein complex. A separate series of studies with the human erythrocyte demonstrated that it contains an N unit that is not complexed with catalytic or receptor units. By a complementation assay with S 49 lymphoma cell variant (designated N^-) that contain a C but not an N unit, it was found that the N unit in the human erythrocyte mediates the actions of guanine nucleotides, fluoride ion, and cholera toxin. Some properties of the N unit were evaluated both in its free and complexed form with the catalytic unit.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Previous studies have shown that the structure of adenylate cyclase systems may be approached by examining the regulatory properties of a receptor (R)-GTP regulatory protein(N) complex (RN) and the complex formed between the catalytic unit (C) and N or RN. Two systems were examined that give promise for further evaluating the role of RN and N in formation of the activate forms of the enzyme. These systems are the turkey erythrocyte cyclase system, and a lymphoma cell membrane (designed N^-) that contains C but not N, and human erythrocyte membranes that essentially lack C but which contain N.

Methods Employed: Turkey erythrocyte, human erythrocyte, and S 49 N^- membranes were isolated by established procedures. Hormone binding and adenylate cyclase assays were performed as described in earlier reports. The details of the reconstitution procedure (complementation assay) are given in a separate report on purification of N from turkey erythrocyte membranes.

Major Findings: (I) Properties of the turkey erythrocyte adenylate cyclase system. It has been reported in other laboratories that catecholamine binding to the beta receptors in turkey membranes is not modified by GTP through the so-called RN complex as is seen with other receptor-cyclase systems. Nor do agonists bind tightly to the turkey beta receptor in the absence of GTP, a general phenomenon observed with other receptors. Thus, either the RN complex is missing in turkey erythrocytes or the N unit is already occupied by a guanine nucleotide such that the properties of the receptor are in the guanine nucleotide mode of behaviour. The latter proved to be the case. Pretreatment of the membranes with isoproterenol plus GMP (a weakly binding guanine nucleotide) followed by washing to remove both hormone and guanine nucleotides resulted in a membrane preparation with markedly differing characteristics: (i) Isoproterenol binding to receptor displayed higher affinities than untreated membranes; (ii) addition of GTP resulted in reduction in affinity for agonists for the receptor, as seen with other receptors; (iii) activation of adenylate cyclase by Gpp(NH)p was greatly enhanced; activation by GTP was transiently increased; (iv) the lag in activation by Gpp(NH)p seen in the absence of hormone is greatly reduced; (v) addition of GTP or GDP to the pretreated system followed by washing results in restoration of the enzyme system to its original characteristics, thus indicating that GTP or GDP become tightly bound to the N unit. All of these findings are compatible with the turkey cyclase system having an N unit that contains tightly bound GDP (the inhibitor at the GTP-regulatory protein). Thus the hormone causes release of bound nucleotide and allows exchange with activating ligands such as Gpp(NH)p or GTP. The latter gives a transient increase probably because it is rapidly hydrolyzed to GDP, thus aborting the activation cycle. We conclude from these findings that the previously demonstrated GTP effects on hormone binding in other systems has its counterpart with an expected effect of hormones on guanine nucleotide binding. These findings provide strong evidence that the negative heterotropic effects of hormone and guanine nucleotides on each other's binding is due to a struc-

ural linkage between N and R units. Thus, the turkey enzyme system, which others have claimed to be a "simplified" version of hormone sensitive cyclase is in fact a complex; the studies reported here suggest that some factor is removed or missing that causes the turkey system to behave "abnormally".

Cholera toxin treatment (with its substrate NAD) caused the turkey enzyme to respond strongly to both GTP and isoproterenol. Our studies revealed that the toxin enhanced the release of bound GDP and exchange with GTP; toxin treatment also made GTP more effective as an activator, suggesting that decreased hydrolysis of GTP to GDP may be one result of the toxin's action. In addition, the toxin decreased the apparent affinity of the activating sites for guanine nucleotides, and markedly repressed the activating effects of fluoride ion. It would appear that the toxin exerts actions on the N unit which result in multiple changes in the properties of the enzyme system. It is, therefore, a valuable tool for investigating the structure and function of the N units responsible for activation of the enzyme.

(II) Complementation analysis: The properties of the N unit when present in the membranes are not easy to evaluate separately since it normally forms complexes with C (or R) that preclude simple analysis. We have found, however, that the human erythrocyte contains an N unit which is effectively unlinked to R or C; the latter are so low (R for catecholamine receptors) that they can be considered to be functionally absent. We have obtained a cell line from S 49 transformed lymphoma cells (termed AC^-) that lacks N unit (thus, we term them N^-) but which contains a C unit that reacts well with MnATP but poorly with MgATP as substrate. Human erythyocyte membranes combined with N^- membranes results, in the presence of either fluoride ion or Gpp(NH)p, in marked enhancement of adenylate cyclase activity in the presence of MgATP as substrate. Parallel increases in the effects of both activators are seen as a function of added H.E. membranes to S 49 N^- membranes. Cholera toxin treatment, also in parallel, increased both GTP-activation and inhibited fluoride activation. Finally, incubation of H.E. membranes with ^{32}P-NAD and toxin gave rise to a single labeled band on SDS acrylamide gel chromatography, the apparent size of the labeled protein is 39,000 daltons or similar to the size of the N unit reported labeled in other cell membranes. We conclude from these findings that the N unit in the human erythrocyte is capable of combining with the C unit in N^- S 49 membranes and is responsible for the activation of adenylate cyclase by Gpp(NH)p, fluoride ion, and cholera toxin. We further evaluated some of the properties of the H.E. N unit. Firstly, preincubation with any guanine nucleotide followed by washing prior to complementation with N^- resulted in no stimulation of cyclase activity. These findings indicated that the N unit uncomplexed with C binds guanine nucleotides weakly and reversibly. However, when the N^- and N^+ membranes are incubated together with Gpp(NH)p, a persistent activated state of the enzyme is formed; this finding is compatible with Gpp(NH)p forming a tight-binding complex when N forms a union with C. Finally, treatment of H.E. N with N-ethylmaleimide irreversibly inactivated the N unit, thus indicating that this unit contains a sulfhydryl group that is essential for complementation with C and possibly for the interaction of guanine nucleotides with the regulatory site on N.

Significance to Biomedical Research and the Program of the Institute: These studies provide new evidence that the nucleotide regulatory units responsible for regulation of adenylate cyclase activity are separate entities from the catalytic unit and from the receptors that mediate hormone action on adenylate cyclase systems. The information gained from studies of the turkey and human erythrocytes N units should greatly facilitate their isolation and characterization.

Proposed Course of the Project: Future studies of the exchange reaction are planned. These will cover such aspects as multi-receptor systems in the exchange reaction, the role of lipids, the sizes of the components, the valence of the catalytic unit, the generality of the process, and its detailed involvement in hormone + GTP activation. Complementation studies will be extended to investigate the molecular basis for the functional interaction between vesicles, and the detailed conditions required to restore a hormone + GTP response.

Publications:

Lad, P.M., Preston, M. Sue, Welton, A.F., Nielsen, T.B. and Rodbell, M.: Effects of Phospholipase A_2 and Filipin on the Activation of Adenylate Cyclase Biochim. Biophys. Acta 551, 368-381, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15001-05 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Structure-function Relationship of Glucagon

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Martin Rodbell	Chief	LNE	NIAMDD
	David E. Wright	Staff Fellow	LNE	NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Glucagon$_{1-2}$ was found to be a partial agonist on the glucagon-sensitive adenylate cyclase system in liver membranes. The potency was 0.001% of the native glucagon molecule. Secretin-amide$_{1-6}$ and VIP$_{1-7}$ which differ from glucagon$_{1-7}$ by having an aspartate residue instead of glutamine at residue 3, failed to interact with the glucagon receptor. It appears, therefore, that residue 3 of glucagon is necessary for selective recognition and action of glucagon. Increased potency of glucagon was obtained with increasing chain length; glucagon$_{1-12}$ is fully active with .05% of the potency of glucagon. Glucagon$_{13-29}$ is inactive as an agonist but competes with glucagon but not with glucagon$_{1-12}$ at the receptor. It is concluded that both specific recognition and activation resides in the N-terminal region whereas the C-terminal region is responsible for tight binding (or potency) of the intact hormone. We have also prepared amidinated lysyl derivatives and tryptophan derivatives of glucagon that are fully active and as potent as the native hormone.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: A major goal of this project is to determine the structural requirements for glucagon binding and action at its receptor. In one sense such knowledge will serve to "map" the domains or regions of the receptor that complement the structure of the hormone. In another, perhaps broader sense, it will assist in determining what is required to modify the glucagon structure such that it will behave as a potent antagonist. A second goal is to prepare affinity analogs of glucagon that can react covalently with the receptor and thus provide a "tag" for isolation and characterization of the receptor.

Methods Employed: Glucagon$_{1-6}$ was obtained from commercial sources and was purified to homogeneity by HPLC. Glucagon fragments 1-12 and 13-29 were prepared by trypsin digestion of native or appropriately modified glucagon; the fragments were purified by gel filtration, ion-exchange chromatography, partition chromatography, and finally to homogeneity by HPLC. Secretin-amide$_{1-6}$ was supplied in its chemically synthesized, purified form. VIP$_{1-7}$ was synthesized by solid-phase procedures. Amidinated derivatives of the glucagon lysyl residue were prepared by reaction with appropriate amidines and purified. Derivatives of the tryptophan residue were prepared by reaction with nitrophenylsulfenyl chloride or by conversion of tryptophan to 2-thiol-tryptophan. All compounds were tested for biological activity either by their effects on adenylate cyclase activity in liver membranes or by competitive displacement of ^{125}I-glucagon binding to glucagon receptors in these membranes.

Major Findings: Previous studies had suggested that removal of but a few of the C-terminal residues from glucagon resulted in complete loss of biological activity. However, we found subsequently that the loss of activity was actually due to marked reduction in affinity for the receptor, thus requiring much higher concentrations of the modified glucagons. This was true for glucagon$_{1-21}$. Most dramatically it was also true for the simple peptide glucagon$_{1-6}$ which proved to be a partial agonist with about 0.001% the potency of native glucagon. This was an important finding since it indicated that essentially all of the biological activity of glucagon resides in the N-terminal residues. As we had previously suggested, potency of binding and action must reside in the residues extending from 7-29. An interesting question was whether not only action but also specificity of hormone binding could be accounted for in the first six or seven N-terminal residues. For the latter question we compared the biological actions and binding of secretin$_{1-6}$ and VIP$_{1-7}$ with that of glucagon$_{1-6}$. The major difference between these derivatives of gastro-intestinal hormones and glucagon is at position 3 which contains aspartate whereas in glucagon this residue is occupied by glutamine. This seemingly minor difference proved decisive since neither VIP or secretin reacted with the glucagon receptor. Thus, both action and specific recognition can be accounted for in the first seven residues of glucagon. We next tested whether a C-terminal fragment, glucagon$_{13-29}$, could compete with native glucagon or with glucagon$_{1-6}$ at the receptor. This compound, as expected, was inactive as an activator of adenylate cyclase. However, it binds with 0.01% the affinity of native glucagon at the

receptor and displaces ^{125}I-glucagon. This derivative did not, however, compete with glucagon$_{1-6}$ for activation of adenylate cyclase. These findings suggest that the receptor contains distinct domains for recognition and binding of the N-terminal and C-terminal regions of glucagon.

Amidinated derivatives of the lysine residue (position 12) of glucagon retain complete biological activity and potency. Previous studies with lysine amide derivatives were either inactive or poorly reactive suggesting that the positive charge is important for the biological actions of the hormone. The amidinated derivatives of lysine that we have prepared contain bulky substitutions that allow the possibility of incorporating photoaffinity analogs. We have also prepared derivatives of the tryptophan residue which are fluorescent and useful for studying the solution properties of the hormone. Recent fluorescent studies indicate that glucagon aggregates in solution at concentrations as low as 10^{-6}M.

Significance to Biomedical Research and the Program of the Institute: Studies of the structure-function relationships in hormones are essential for understanding the mechanism of action of hormones at their receptors and for understanding the physical properties of these biologically important agents. Potentially such information can give insights for the chemical synthesis of new forms of hormones that are either more active or are antagonists. Both types could be valuable for clinical investigation and perhaps for ameliorating endocrine disturbances.

Proposed Course of Project: We are preparing several synthetic analogs of the active N-terminal region of glucagon with the aim of determining the minimal changes that result in loss of biological activity. In addition, substitution of D-amino acid residues for certain residues in the N-terminal region will be made as a possible means of preparing antagonists. The photoaffinity analogs of the hormone prepared by modification of the lysine and tryptophan residues will be employed for covalent incorporation at the glucagon receptor with the aim of isolating the receptor.

Publications:

Wright, David E., Hruby, Victor J., and Rodbell, Martin: A Reassessment of Structure-Function Relationships in Glucagon: Glucagon$_{1-21}$ is a full agonist. J. Biol. Chem. 253: 6338-6340, 1978.

Wright, David E. and Rodbell, Martin: Glucagon$_{1-6}$ binds to the glucagon receptor and activates hepatic adenylate cyclase. J. Biol. Chem. 254: 268-269, 1979.

Wright, D.E. and Rodbell, M.: Gastrointestinal hormone-receptor recognition: position three is a determining residue for glucagon, secretin, and VIP. Proc. Sixth Amer. Peptide Symposium, 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOI AM 15004-04 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies of Hormone Responses in Cultured Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael C. Lin Research Chemist LNE NIAMDD

COOPERATING UNITS (if any)

Dr. Robert T. Simpson, LNE NIAMDD
Dr. Lois Tice, LEP NIAMDD

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	none

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Conditions required for expression of hormone (glucagon or epinephrine) action on adenylate cyclase systems have been examined with cells in culture. Primary rat adult hepatocytes fail to grow in culture but maintain hormone responsiveness provided that insulin and glucocorticoids are present during culture. By contrast, cells (fetal hepatocytes, HeLa, erythroleukemic) that grow in culture media display poor responsiveness to hormones unless differentiation-inducing agents (butyric acid, dimethylsulfoxide, 1-methyl-3-isobutylxanthine) are present. The actions of these agents do not involve effects on the cell cycle.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The goals of this project are: 1) To study the changes in responsiveness of cell cultures to hormones such as epinephrine and glucagon using adenylate cyclase activity and hormone binding to receptors as criteria; 2) To elucidate the biochemical basis for these changes; and 3) to investigate the relationship between cell differentiation and growth on the regulation of hormone-responsiveness.

Methods Employed: Hepatocytes were isolated from collagenase-treated adult and fetal livers by conditions established in other laboratories. Erythroleukimic and HeLa cells were established cell lines. All cells were cultured in media containing serum and in some cases with hormone-enriched media. Effects of hormones (glucagon or epinephrine) were determined 1) by measuring cyclic AMP formation in intact cells (using cyclic AMP immunoassay procedures), 2) after homogenization and assays for adenylate cyclase activity by procedures established in this laboratory, and 3) by investigating the binding of ^{125}I-glucagon or ^{125}I-hydroxybenzylpindolol (or ^{3}H-alprenolol) to beta adrenergic receptors. Other hepatic functions, such as albumin and a-fetal protein, were measured by immunoassay employing standard procedures. Cell growth was quantitated with a cell counter; incorporation of ^{3}H-thymidine used as an index of DNA synthesis.

Major Findings: Hepatocytes from adult rat liver were found to become rapidly insensitive to glucagon and epinephrine after incubation in culture media. However, when incubated in culture media enriched with insulin and hydrocortisone, the appearance of both glucagon- and epinephrine-stimulated adenylate cyclase activities appeared within 4 hrs and increased steadily until these activities reached maximum (2 to 3 days). Prostaglandin, a relatively weak activator of hepatic adenylate cyclase also increased but required 5 to 7 days for full restoration of responsiveness. The primary cultures remained responsive to all three hormones for at least 7 weeks in the culture medium. Cell growth was minimal under conditions required to maintain hormone responses.

The loss of hormone response found shortly after culturing the cells was not due to proteases, such as plasminogen activator, which are known to be released from cells during culture. Protease inhibitors failed to prevent loss of hormone responsiveness. Similar findings were recorded with all cultured cells (HeLa, Fetal liver, and leukemic cells). These cells, in contrast to adult hepatocytes grow rapidly in serum-enriched media but displayed minimal levels of either epinephring or glucagon responsiveness. However, addition of known differentiation-inducing chemicals (butyrate, MIX, and dimethylsulfoxide) caused dramatic increases in hormonal responses while inhibiting growth of the cells. Increased hormone-responsiveness was associated in the case of HeLa cells, with an increase in the concentration of beta adrenergic receptors on the cell membrane. A detailed investigation of the relationship of the cell cycle to the effects of these agents hormonal responsiveness failed to show a correlation between these processes. It would appear, therefore, that the cell cycle, though affected by these agents, does not influence as best as can be

determined by current methodology, the appearance of epinephrine receptors or of epinephrine activation of adenylate cyclase.

Significance to Biomedical Research and the Program of the Institute: One of the means by which the cell is capable of interacting with its enviroment is to have recognition sites or components on its cell membranes that allows extracellular agents to interact with the cell. Hormone receptors represent a class of molecules which help to differentiate cell types. Thus, it is essential to understand what processes determines the insertion of selective hormonal responsiveness of cells. In this respect, studies of the relationship between cellular growth and differentiation may serve to determine what role hormone receptors have in the selection, differentiation, and growth processes.

Proposed Course of the Project: We intend to investigate the fate (turnover) of receptors for glucagon and catecholamines during cellular differentiation and growth. These studies will be facilitated with the use of analogs of these hormones that interact covalent with their receptors. Such analogs have been prepared and display promising signs of giving information both at the biochemical and electron microscopic level. In addition, as other components (nucleotide regulatory and catalytic) become amenable for quantification, these units will be investigated. The latter should assist to understand how these various units are incorporated in the membrane and what influences growth and differentiation processes have on their turnover in the cell.

Publications

Lin, Michael C., Welton, A.F. and Berman, M.F.: Essential role of GTP in the expression of adenylate cyclase activity after cholera toxin treatment. J. Cyc. Nuc. Res. 4: 159-168, 1978.

Lin, Michael C., Lin, Chiu-shiong and Whitlock, J.P.,Jr.: Reduction of GTP activation of adenylate cyclase system by its coupling to hormone receptor. J. Biol. Chem. 254: 4684-4688, 1979.

Lin, Chiu-shiong and Lin, Michael C.: Appearance of β-adrenergic response of adenylate cyclase during the induction of differentiation in cell cultures. Exp. Cell Res. 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOI-AM 15005-04 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Actions of Adenosine on Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. Londos	Research Chemist	LNE	NIAMDD
Other:	D.M.F. Cooper	Visiting Fellow	LNE	NIAMDD
	W. Schlegel	Visiting Fellow	LNE	NIAMDD
	D. Wright	Staff Fellow	LNE	NIAMDD

COOPERATING UNITS (if any)

J. Wolff Chief, CEB NIAMDD

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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1.0	1.0	none

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In previous studies we have established that plasma membranes of most cells contain adenosine receptors through which the nucleoside activates adenylate cyclase, but that the fat cell receptor mediates inhibition. With the use of dATP as the cyclase substrate, we now find that analogs, particularly N^6-phenylisopropyladenosine (PIA) and 5'-N-ethylcarboxamindeadenosine (ECA), clearly distinguish between activatory and inhibitory receptors. Activation of liver and Leydig tumor cell cyclases exhibits the potency series: ECA > Adenosine ≥ PIA, whereas the potency series for inhibition of the fat cell enzyme is : PIA > adenosine > PLA. Moreover, the respective potency series are maintained in physiologic studies with intact cells. These studies show that distinct subclasses of adenosine receptors exist, and underline the predictive value of the use of adenylate cyclase as a probe for development of pharmacologic agonists and antagonist of the adenosine receptor.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: We have found that many adenylate cyclase systems contain adenosine receptors which mediate increased activity. On the other hand, the fat cell enzyme contains a receptor which mediates inhibition of cyclase activity. Although activatory and inhibitory receptors in isolated membranes exhibit superficial similarities in their reactions with agonists and antagonists, physiologic data with intact cells suggest that adenosine receptors are not homogeneous. However, interpretation of pharmacological studies on intact cells is made difficult because of the possibility of uptake or metabolism of nucleosides. One goal of these studies was to develop an adenosine-free adenylate cyclase assay, and to then examine the pharmacology of adenosine receptors in purified membranes in order to determine whether there exist distinct subclasses of adenosine receptors.

Methods Employed: An adenylate cyclase assay methods was developed in chich deoxy ATP (dATP) was employed as substrate. We had found in previous studies that deoxyadenosine is a poor effector at adenosine receptors; thus production of deoxyadenosine from dATP should not interfere with the actions of adenosine or other analogs added to the medium. The assay previously developed in this laboratory for measuring cyclic AMP had to be modified for the assay of deoxycyclic AMP produced from labeled dATP. In all other respects, the studies reported were carried out with techniques established in this laboratory.

Major Findings: Two functionally distinct adenosine receptors were identified with the use of adenosine and two analogs: N^6-phenylisopropyladenosine (PIA) and 5'-N-ethylcarboxyamideadenosine (ECA). The following order of potency of these compounds on liver and Leydig tumor cell membrane adenylate cyclase activity (stimulatory) was found: ECA > adenosine > PIA. By contrast, the inhibitory adenosine receptor in fat cells showed the potency series: PIA > adenosine > ECA. Thus, based on pharmacological evidence it appears that the receptors for adenosine that stimulate and inhibit adenylate cyclases are structurally distinct. Moreover, these potency series were maintained for stimulation of steroidogenesis in Leydig cells and for inhibition of lipolysis in isolated rat adipocyte.

Another important distinction of the adenosine inhibitory receptor, as opposed to stimulatory receptors, is the role of sodium ions on activity in isolated membranes. In the case of the fat cell adenylate cyclase, inhibition is markedly enhanced by two ligands, GTP and sodium; potassium and choline exert relatively weak effects, suggesting that the cation site is selective. Mild trypsin treatment of intact adipocytes altered the response to sodium ions. Thus, it appears that sodium sites are involved in the process through which GTP and adenosine inhibit the fat cell adenylate cyclase.

Significance to Biomedical Research and the Program of the Institute: Adenosine acts at cell surfact receptors to mediate a wide variety of biologic effects, such as coronary artery dilation, suppression of immune function in lymphocytes, and potentiation of histamine release in mast cells. Considerable

effort is now being put forthe in the search for pharmacologically active agonists and antagonists of the adenosine receptor(s). Our studies reveal that there are distinct subclasses of adenosine receptors. Moreover, the methods we have developed provide a means for rapid testing and screening of pharmacologic agents.

In contrast to hormones which increase adenylate cyclase activity, hormones which decrease activity seem to exert a variety of effects, such as modifying ion movements across membranes and increasing phospholipid turnover, which may be independent of their effects on cyclase. However, an understanding of the mechanism of signal transduction by "inhibitory" hormone receptors, with the use of adenylate cyclase, may provide clues for examining, in isolated membranes, the adenylate cyclase-independent actions of such receptors.

Proposed Course of the Project: Further studies will involve: 1) biochemical identification of adenosine receptors with the use of radiolabeled photoaffinity adenosine analogs; and 2) with the use of knowledge gained from our studies on the inhibitory effect of adenosine on the fat cell cyclase, an attempt will be made to resolve the controversial question as to the actions of insulin on adenylate cyclase.

Publications

Londos, C., Cooper, D.M.F., Schlegel, W. and Rodbell, M.: Adenosine analogs inhibit adipocyte adenylate cyclase by a GTP-dependent process: Basis for actions of adenosine and methylxanthines on cyclic AMP production and lipolysis. Proc. Nat. Acad. Sci. USA 75: 5362-5366, 1978.

Wolff, J., Londos, C. and Cook, G.H.: Adenosine interactions with thyroid adenylate cyclase. Arch. Biochem. Biophys. 191: 161-168, 1978.

Londos, C., Wolff, J., and Cooper, D.M.F.: Actions of adenosine on adenylate cyclase. In: Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides. (Eds. H.P. Baer and G.I. Drummond) Raven Press (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOI AM 15006-04 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of the GTP-regulatory Component of Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.B. Nielsen	Staff Fellow	LNE	NIAMDD
Other:	P.M. Lad	Sr. Staff Fellow	LNE	NIAMDD
	M.S. Preston	Biologist	LNE	NIAMDD

COOPERATING UNITS (if any)

A. Spiegel, R. Downs, G. Aurbach NIAMDD

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Laboratory of Nutrition and Endocrinology

SECTION
Section on Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The first stages in the large scale purification of the GTP-regulatory protein involved in activation of adenylate cyclase have been carried out. A simple system using S 49 lymphoma cells lacking this protein has been developed which allows rapid assays of N on a microscale either with neutral detergent extracts of membranes containing N units or even with intact membranes. Turkey erythrocyte membranes proved to have a sufficiently high concentration of N units for purification studies. Modest purification has been achieved on a GTP-sepharose affinity column.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: A major objective of this study is to purify the GTP-regulatory protein (abbreviated N) which is responsible for activation of adenylate cyclase by guanine nucleotides, fluoride ion, and cholera toxin. It may also by responsible for linking various hormone receptors to the enzyme system.

Methods Employed: Several procedures had to be developed for purification of N. GTP-Sepharose affinity columns were prepared in Dr. Aurbach's laboratory and proved effective for concentrating detergent-solubilized extracts of turkey erythrocyte membranes, the major source of N for this project. Assay for N was essentially that developed by Ross and Gilman and utilized the fact that an S 49 lymphoma cell line (termed ac^-) lacks an N unit (we term it, therefore, an N^- mutant) but contains a catalytic unit which does not respond to any known activator of the enzyme present in normal membranes. When a detergent extract containing N is added to the N^- membranes, activation by Gpp(NH)p, fluoride ion, and cholera toxin (plus GTP and NAD) takes place. This "complementation" assay also works simply by mixing membranes containing N with the N^- membranes along with assay ingredients and appropriate activating agents (Gpp(NH)p or fluoride ion). This assay procedure proved decisive for evaluating the concentration of N units in various membrane preparations. The turkey erythrocyte membrane was chosen because of its plentiful supply, its richness in N units, and the fact that after appropriate treatment of the membranes prior to detergent solubilization, the N unit was retained on GTP-agarose affinity columns while the catalytic unit passed through. After eluting the N units with a guanine nucleotide agonist the concentration of N unit was assayed by the complementation assay. This procedure proved invaluable for concentrating N units on a relatively large scale; the complementation assay was essential for analyzing the presence of functional N units. Although the success of this project will depend on large scale processing of turkey erythrocyte N units, another critical facet of the project is large scale production of N^- membranes from S 49 ac^- cells. Thus far, we have satisfactorily processed these membranes from 50 liter batches of the cells.

Major Findings: The major success thus far is in finding that GTP-sepharose affinity chromatography results in at least a 100-fold purification. More importantly, it provides the means of concentrating N units from detergent extracts and has the advantage of removing detergents prior to further purification of this material.

Significance to Biomedical Research and the Program of the Institute: The GTP-regulatory protein involved in guanine nucleotide and hormonal regulation of adenylate cyclase is an important linkage in the chain of events leading to hormone action in cells. By purifying the material and understanding its chemistry and functions, the actions of hormones will be better understood.

Proposed Course of the Project: Other purification steps, including hydrophobic column chromatography, DEAE-Sephadex chromatography, and Thioagarose chromatography are but a few of the schemes being developed. We also plan to obtain antibodies against N units as soon as sufficient material is available for injection into rabbits or mice. If the material proves antigenic and good antibody production is obtained, a valuable new tool for concentrating, purifying, and even localizing N units in membranes will be available.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOI AM 15007-01 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Target Size Analysis of Adenylate Cyclase and Hormone Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Werner Schlegel	Visiting Fellow	LNE	NIAMDD
	Martin Rodbell	Chief	LNE	NIAMDD
Other:	Thor B. Nielsen	Staff Fellow	LNE	NIAMDD
	Pramod M. Lad	Sr. Staff Fellow	LNE	NIAMDD
	Dermot M.F. Cooper	Staff Fellow	LNE	NIAMDD
	Michael C. Lin	Research Chemist	LNE	NIAMDD
	M. Sue Preston	Biologist	LNE	NIAMDD

COOPERATING UNITS (if any)

E.S. Kempner LPB, NIAMDD

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Membrane Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Target size analysis, using high energy electron radiation, was employed to estimate the functional target sizes of the adenylate cyclase systems in plasma membranes of liver, adipocytes, and turkey erythrocytes. The functional sizes of both liver and adipocyte adenylate cyclase increased with complexity of regulation, suggesting that the regulatory (hormone receptors and GTP-regulatory proteins) become linked to the catalytic unit when put in their activated states by hormones, guanine nucleotides, and fluoride ion. Prior to "coupling" the regulatory units are "multimers" of the units attached to the catalytic unit (the activated holoenzyme). By contrast, multimeric regulatory units were not detectable in turkey erythrocyte membranes; this system seems to be pre-assembled as the holoenzyme even prior to activation by catecholamines and guanine nucleotides. The regulatory units responsible for inhibition of the adipocyte cyclase system by GTP and adenosine yield a target size prior to coupling that is considerably larger than the multimeric "activating" units, thus supporting previous findings that inhibition and activation of adenylate cyclase by GTP are carried out through separate molecular structures.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Hormonal regulation of adenylate cyclase systems involves at least three classes of proteins: hormone receptors, GTP-regulatory proteins, and the catalytic unit that converts ATP to cyclic AMP. GTP-regulatory proteins can be divided into two types, a stimulatory type which is coupled to numerous peptide hormones, biogenic amines, and prostaglandins and an inhibitory type which is linked, in the case of the fat cell system, to adenosine receptors. A fundamental question is how the various regulatory units (hormone receptors and GTP-regulatory proteins) are assembled before and after being liganded. The only method currently available that can give such information is that of target analysis by high energy electron radiation. This technique measures the functional size of macro molecules even in impure preparations such as is the case of adenylate cyclase systems in plasma membranes. The objective of this study is to evaluate the target sizes of the liver, adipocyte, and turkey erythrocyte systems both before and after exposure to the various ligands that either stimulate or inhibit cyclase activity.

Methods Employed: Liver, adipocyte, and turkey erythrocyte membranes were isolated by previously established methods and were analysed for adenylate cyclase activity by methods developed in this laboratory. In some cases binding of ^{125}I-glucagon was used to measure the quantity of glucagon receptors present in liver plasma membranes; GTP-displaceable glucagon binding was used for determination of the complex formed between the hormone receptor and the GTP-regulatory unit (referred to as the RN unit). The membranes (frozen and maintained at -110° during radiation) were irradiated with high energy electrons generated by a 15 MeV electron accelerator (Naval Research Labs). Semi-log plots of surviving adenylate cyclase activity (or glucagon binding activity) versus radiation dose yielded, with appropriate corrections, the functional sizes of these units. Assays of activity were carried out either following irradiation; i.e., the "ground-state" sizes prior to activation (or inhibition) of the enzyme, or in the "activated" state in which case the membranes were exposed to various activating ligands prior to irradiation. The latter gives an estimate of the functional "activated" target size of the enzyme system.

The functional size of the GTP-inhibitory process in fat cell membranes was evaluated by comparing the radiation dosage necessary to destroy the stimulatory and inhibitory effects of GTP which occur over differing concentrations ranges of the nucleotide. Because of the sizes of the GTP-inhibitory and stimulatory processes overlapped, it was not possible to obtain an absolute target size of these processes in fat cells.

Major Findings: Irradiation of liver adenylate cyclase prior to activation; i.e., the ground state enzyme, displayed a non-linear relationship between surviving cyclase activity and radiation dosage. Assuming two major targets, the slopes of the radiation curves yielded targets of approximately 10^6 and 2.3×10^5 daltons. However, when the enzyme system was placed in its pre-activated states by treatment with fluoride ion, Gpp(NH)p, or with glucagon plus GTP, the larger component disappeared and single exponentials were obtained. The target sizes observed with fluoride ion and Gpp(NH)p were the

same as the smaller size of the "ground-state" enzyme (2.3×10^5 daltons), whereas the size of the enzyme system activated by glucagon plus GTP was 3.5×10^5 daltons. These findings suggested that the holoenzyme responsive to glucagon and GTP is comprised of larger units than the enzyme linked to guanine nucleotide (Gpp(NH)p) or fluoride activation. We concluded that the difference in size is due to the linkage of the receptor (R) to the enzyme system in the activated state. Previous studies had shown that MnATP is the preferred substrate for the catalytic unit (C) whereas the catalytic unit linked to the GTP-regulatory unit (N) can use MgATP or MnATP as substrate. We therefore estimated the target size of the "ground-state" enzyme using MnATP as substrate and no added activating ligands. The larger target, though still observed, was considerably less (at most 20%) in terms of the percentage of total activity that was seen with MgATP as substrate. More to the point, the size of the smaller unit was about 1.5×10^5 daltons; i.e. considerably smaller than the ground state unit measured with MgATP (2.3×10^5 daltons). We concluded from these findings that a large percentage (about 70%) of the enzyme activity in the ground state is represented by the catalytic unit (C) uncomplexed with the GTP regulatory units; the remaining 30% is an NC unit through which guanine nucleotides and fluoride ion activate the enzyme. Activation by hormone plus GTP yields the holoenzyme (RNC). Since all of the activity of the enzyme can be accomodated by target sizes of 350,000 daltons or less, it was apparent that the larger target of the ground state enzyme must represent regulatory units (R,N) that are present as a large complex and which must breakdown during incubation to yield smaller units that can form complexes with C. These large complexs could either be aggregates of R and N or aggregates of complexed RN (termed $(RN)^n$). Target analysis of the GTP-displaceable ^{125}I-glucagon binding components in liver membranes yielded a single exponential decay curve and a molecular weight in the range of 8×10^5 daltons. This size is comparable to the size of the large ground state target (10^6 daltons). We conclude, therefore, that the RN complex is likely to be the ground state target and that is pre-exists in the membrane as a multimer $(RN)^n$. Activation by hormone and GTP involves the binding of these ligands to their respective monomeric units (RN) that complex either with the catalytic unit (C) to give RNC, with NC to give RNNC, or with both. Thus target analysis of the liver enzyme system has provided a powerful tool for determining whether the regulatory and catalytic units exist separately or linked prior to liganding. The results suggest that these units are largely uncoupled prior to activation.

Target analysis of the adipocyte adenylate cyclase system yielded the identical patterns of sizes seen with the hepatic enzyme when activating ligands (Gpp(NH)p, fluoride, or hormones (ACTH, isoproterenol, glucagon) plus GTP) were used in the assay. The remarkable similarity in the sizes of the two enzyme systems suggests that the hormone receptors and nucleotide activating units are comparable in size (and, of course, in function).

A different ground-state target size developed when inhibitory concentrations of GTP were present in the assay medium. Thus, for example, when an inhibitory concentration of 10^{-6} M GTP was employed, inhibition of enzyme activity was more susceptible to loss in function with radiation dosage relative

to that seen with stimulatory concentrations (10^{-8} M in the presence of activating hormones). These findings indicate that the GTP-inhibitory process contains a different structure than the process through which GTP and hormones activate the enzyme. The larger structure of the inhibitory process may be composed of both the adenosine receptor and the GTP-inhibitory unit since our previous studies have established that the GTP-inhibitory process is promoted by adenosine.

Target analysis of the turkey erythrocyte, although still in a preliminary stage, has given valuable insights as to why this system behaves kinetically dissimilar to either the liver or adipocyte cyclase systems. Target analysis of either the ground state or the GTP plus isoproterenol-activated state of the turkey system gave the same functional target size (about 3.5×10^5 daltons); i.e., the larger targets (presumably multimeric regulatory units of RN) of the latter were not observed with the turkey system. Further analysis is necessary to determine whether 3.5×10^5 daltons represents the RNC unit seen, for example, in the liver. Interestingly, only a very small percentage of the C unit (1.5×10^5 dalton) is seen in the turkey membranes. It thus seems likely that the turkey enzyme pre-exists in the membrane as the holoenzyme; i.e., the regulatory units are already coupled to the catalytic unit. This may account for the major differences in kinetic characteristics of the turkey compared to other adenylate cyclase systems that we and others have examined.

Significance to Biomedical Research and the Program of the Institute: Adenylate cyclase is a key enzyme system in the actions of numerous hormones and neurotransmitters. How the various hormones modify - either by inhibiting or stimulatory activity - the enzyme is a major question in biology. The studies summarized here provide the first tangible evidence that the various regulatory components (hormone receptors and nucleotide regulatory units), which we have established previously to be essential in hormone and GTP action, can exist separately from the enzyme in the native environment of the cell membrane. We can now begin to understand the basis of the efficiency with which hormones act on these systems based on the assumption that the regulatory units are composed of multimeric subunits, probably organized as a unit in the membrane. The finding that the GTP-inhibitory process in the adipocyte is a separate structure from the GTP-activating process provides another important piece of information regarding the complex nature of the regulation of adenylate cyclase systems and should assist in evaluating the actions of numerous compounds known to inhibit adenylate cyclase in various types of cells.

Proposed Course of the Project: Target analysis will be continued but with particular emphasis on determining the sizes of the receptors and nucleotide regulatory components employing binding of hormones and guanine nucleotides as functional activities. We also plan to determine the target sizes of other membrane regulatory enzymes such as the Na-K ATPase which is the key system for transport of solium and potassium ions in membranes.

Publications

Rodbell, M. and Schlegel, W.: The Role of GTP in the Coupling of Hormone Receptors and Adenylate Cyclase. In Juxe, K., Hokfelt, T. and Luft, R. (Eds) Central Regulation of the Endocrine System. Plenum Publishing Corp., 1979, pp. 71-83.

Schlegel, W., Kempner, E. and Rodbell, M.: Activation of Adenylate Cyclase in Hepatic Membranes Involves Interactions of the Catalytic Unit with Multimeric Complexes of Regulatory Proteins. Journal of Biological Chemistry (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15010-01 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Biphasic Effects of GTP on Fat Cell Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dermot M.F. Cooper	Visiting Fellow	LNE	NIAMDD
Other:	Werner Schlegel	Visiting Fellow	LNE	NIAMDD
	Michael Lin	Research Chemist	LNE	NIAMDD
	Constantine Londos	Sr. Staff Fellow	LNE	NIAMDD
	David E. Wright	Staff Fellow	LNE	NIAMDD
	Martin Rodbell	Chief	LNE	NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The dualistic role of GTP on fat cell adenylate cyclase has been established. Low concentrations (< 20 nM) of GTP activate the enzyme, while higher concentrations cause progressive inhibition, yielding a biphasic GTP titration. The biphasic behaviour is independent of assay conditions, such as time, temperature and metal ion concentration.

Cholera toxin treatment of membranes or trypsin treatment of intact cells results in the abolition of the GTP inhibitory phase, while mercurial treatment causes the selective loss of the GTP activatory phase. Treatments which result in the loss of GTP inhibition also display no sensitivity to inhibition by adenosine or its purine-modified analogs. Such studies suggest that separate processes mediate the biphasic response of fat cell membranes to GTP: the function of the activatory phase is to amplify the stimulatory effects of hormones; the function of the inhibitory process is to amplify the inhibitory effects of adenosine. Preliminary results indicate that monovalent cations [$Na^+ > K^+ > Li^+$] greatly enhance the inhibitory actions of adenosine.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Previous studies had demonstrated that GTP may both activate and inhibit adipocyte adenylate cyclase. The response to GTP was difficult to predict since chelators and sulfhydryl reagents, commonly included in the assay of adenylate cyclase, modified the inhibitory effects of the nucleotide. Contamination of the ATP substrate with GTP made the problem more complex. At "inhibitory" GTP levels we had previously shown that adenosine inhibited activity.

The purpose of the present study was i) to define clearly the degree of certainty with which we might predict the dual effects of GTP and how this behaviour might be modified by assay condition, ii) to determine whether there was any intrinsic requirement by the inhibitory effects mediated by adenosine for the GTP inhibitory behaviour, iii) whether one or two processes were responsible for the dualistic behaviour and what molecular insights might be gained on the system.

Method Employed: Plasma membranes were purified from isolated rat epididymal fat cells by procedures developed in this laboratory. Adenylate cyclase was assayed with α-^{32}P ATP substrate by our routine procedure.

Major Findings: Using purified ATP as substrate we have detected a very sensitive activatory response of the fat cell enzyme to nanomolar concentrations of GTP, which is followed at higher concentrations (> 20 nM) by a decline in activity, yielding a biphasic GTP titration curve. This behaviour is maintained under a variety of assay conditions; it is largely independent of the hormone, assay time, assay temperature and Mg^{2+} concentration. Mn^{2+} ions, however, abolish the inhibitory effect.

We have found that this biphasic behaviour can be modified by a number of treatments; viz. treatment of either plasma membranes with cholera toxin or of intact adipocytes with trypsin results in the loss of the inhibitory phase while the activatory effects of GTP are fully retained. By contrast, treatment of membranes with the mercurial p-hydroxymercuriphenylsulphonate (MPS) activates the enzyme and eliminates the activatory phase while retaining the inhibitory phase. The ability to thus observe either phase of GTP action in isolation suggests the existence of separate processes.

We have also found that conditions which cause the loss in the inhibitory effects of GTP (outlined above) also result in the loss of the ability of adenosine to inhibit the enzyme. Thus the inhibitory phase of GTP action must be demonstrably apparent to observe the effects of adenosine.

The possibility has also been investigated that either generation of GDP into the assay medium or a slow release of GDP from the nucleotide site might be responsible for the inhibitory response to high concentrations of GTP. Using APP(NH)P as substrate and hydrolysis-resistant GDP analogs we have found no selective effect of GDP on either phase of the GTP titration.

Significance to Biomedical Research and the Program of the Institute: It is now becoming apparent that a number of physiological inhibitory substances, including α-adrenergic agents and opiates as well as adenosine exert their effects via processes requiring GTP inhibition. Our studies on the fat cell provide a molecular basis against which such systems can be evaluated and will allow clarification of the mode of action of these agents.

Proposed Course of Project: i) The effects of the full range of GTP concentrations on the binding of catecholamines to the β-receptor will be examined to evaluate how the altered expression of the nucleotide action on adenylate cyclase is transmitted to the receptor. ii) The interaction between the adrenergic and the adenosine receptor will be examined to determine whether occupancy of the adenosine receptor causes decreased affinity at the adrenergic receptor and vice versa iii) The modulation of the response to GTP by alterations in the membrane redox potential will also be investigated as a probe for the mechanism of action of insulin. Preliminary results indicate that the biphasic GTP titration in the fat cell is a sensitive probe for the redox state of the membrane. iv) Search for the generality of this phenomenon as a means whereby inhibitory ligands exert their effect on cellular cyclic AMP production.

Publications:

Londos, C., Cooper, D.M.F., Schlegel, W. and Rodbell, M.: Adenosine analogs inhibit adipocyte adenylate cyclase by a GTP-dependent process: Basis for actions of adenosine and methylxanthines on cyclic AMP production and lipolysis. Proc. Nat. Acad. Sci. USA: 75, 5362-5366, 1978.

Cooper, D.M.F., Schlegel, W., Lin, M. and Rodbell, M.: The fat cell adenylate cyclase system: Characterisation and manipulation of its bimodal regulation by GTP. J. Biol. Chem. 254, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 15011-01 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (30 characters or less)

Regulation of Human Platelet Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dermot M.F. Cooper	Visiting Fellow	LNE NIAMDD
	Martin Rodbell	Chief	LNE NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A purified plasma membrane preparation of human platelets has been obtained, which is essentially free of contaminating catecholamines and nucleotides. In this preparation we have studied the regulation of the adenylate cyclase activity by α-adrenergic agents, PGE_I, GTP and ADP. PGE_I stimulation is greatly enhanced by increasing concentrations of GTP, while α-adrenergic inhibition of activity is totally dependent on relatively high concentrations of GTP.

ADP, a primary physiological inducer of platelet aggregation and potent inhibitor of cyclic AMP production in intact cells, has been shown to inhibit adenylate cyclase activity in this preparation with a K_i of ca. 0.5 μM. Hitherto, such inhibition has not been demonstrated, because of generation of ADP in the adenylate cyclase assay and because of contaminating ADP in membrane preparations. Two other ADP analogs, ADP-S >> $AP(CH_2)P$ also inhibit the enzyme

PHS-6040
(Rev. 10-76)

Project Description

Objectives: ADP, acting extracellularly, is well known to be both a potent inhibitor of Cyclic AMP accumulation in intact platelets and a potent inducer of platelet aggregation. However to this date, no inhibition of adenylate cyclase activity in membrane preparations by this nucleotide has been detected. The problem is made difficult by the ubiquitous presence of phosphohydrolases in membrane preparations, which hydrolyse the substrate, ATP to ADP. Additionally platelet membrane preparations are generally highly contaminated with granules containing ADP, ATP and catecholamines.

The purpose of this study was i) to obtain a membrane sufficiently uncontaminated to permit a search for ADP effects ii) to observe the interaction between PGE_I, α-adrenergic agents and GTP in the absence of ADP iii) to probe for similarities between this system and that of the fat cell, where adenosine inhibits activity and iv) to determine whether ADP does in fact inhibit platelet adenylate cyclase activity.

Methods Employed: Platelet membranes were prepared by a glycerol lysis method followed by sucrose gradient centrifugation. Adenylate cyclase activity was determined using α-^{32}P-APP(NH)P as substrate in the absence of a regenerating system over short incubation times.

Major Findings: A marked enhancement of PGE_I stimulation of adenylate cyclase activity is seen on the addition of low concentrations of GTP, which we take to reflect relatively little contamination of the membrane preparation with GTP. A monophasic titration is observed with increasing GTP concentration in the presence of PGE_I. In the presence of epinephrine the titrations coincided at low GTP concentrations of GTP but diverged at higher ($> 10^{-7}$M) concentrations, giving up to 50% inhibition. This effect was reversed by phentolamine (10^{-5} M). We note the similarity between the dependency for elevated GTP concentrations to observe the effect of α-adrenergic inhibition in this system and inhibition by adenosine in the fat cell system.

Careful titration with ADP shows biphasic inhibition of adenylate cyclase activity in these membranes. In the first phase, activity is inhibited by 25% by concentrations up to 2 μM (app K_i; 0.5 μM), a plateau follows before activity is again reduced by higher concentrations (> 15 μM). The latter phase probably reflects substrate competition (app K_i: 100 μM). The analogs $AP(CH_2)P$ and ADP-S share these effects of ADP with about the same potency. The potent ADP inhibition of platelet adenylate cyclase thus appears to provide the mechanism whereby ADP decreases cyclic AMP production in intact cells.

Significance to Biomedical Research and the Program of the Institute: This first demonstration of the effect of an adenine nucleotide at a putative receptor in an isolated membrane preparation will allow the examination of other systems (brain and intestinal muscle) thought to be modulated by such receptors.

Proposed Course of Project: The modulatory effects of ADP on the actions of the other agents which affect platelet adenylate cyclase will be examined. More potent ADP analogs are being sought to facilitate the identification of such receptors and to evaluate their effects in other systems. Additionally it is hoped to identify specific antagonists of this receptor.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 15100-09 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Protein-Nucleic Acid Interactions: Chromatin Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert T. Simpson	Medical Director (R) (T)	LNE	NIAMDD
Others:	Arnold Stein	Senior Staff Fellow	LNE	NIAMDD
	Peter Künzler	Visiting Fellow	LNE	NIAMDD

COOPERATING UNITS (if any)

Laboratory of Chemical Physics, NIAMDD

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2.5	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Crosslinking and direct binding studies have confirmed our previous suggestion that chromatin core particles can bind excess histones as octamers in 0.6 M NaCl. Acidic proteins, such as polyglutamic acid, also bind histones as octamers, even at physiologic ionic strength. This interaction allows the complete reassembly of SV-40 minichromosomes in a cooperative fashion in less than 60 min, a marked contrast to slower and incomplete assembly in the absence of the polyanion. We have constructed chromatin and core particles from the four smaller histones and synthetic polydeoxyribonucleotides. The complementary homopolymers do not form nucleosomes. The alternating copolymers form chromatin very similar to native material. Due to lack of sequence heterogeniety in the DNA, core particles derived from these chromatins are ideally suited for high resolution studies of histone-DNA interactions. A number of features of core particle structure, conformational transitions, and DNA geometry have been revealed or defined with much higher precision using these semi-synthetic core particles.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To study the structure of chromatin, the DNA histone complex of eukaryotic cells. To define the mechanism of packaging of DNA in the cell nucleus and to determine the mechanisms which lead to stable repression of much of the genetic information in differentiated cells and expression of that portion of the information appropriate for the function of the cell.

Methods Employed: Nuclei are prepared from chicken erythrocytes, rodent liver, or Hela cells, labeled with ^{3}H-thymidine or unlabeled. Nuclear suspensions in isotonic sucrose containing 0.1 - 1 mM $CaCl_2$ are digested for varying lengths of time with micrococcal nuclease and nucleosomes isolated by sedimentation on isokinetic sucrose gradients.

Histones were isolated from chicken erythrocyte chromatin by dissociation from DNA in 2.5 M NaCl, removal of DNA by sedimentation or adsorption onto hydroxyapatite, and purification by gel filtration. The inner histones (H2A, H2B, H3 and H4) were reassociated with various DNA's by either salt step dialysis (from 2.5 M NaCl to 0.1 M NaCl) in the case of the synthetic polydeoxyribonucleotides or by direct mixing in 0.1 - 0.2 M NaCl in the case of the various forms of SV-40 DNA.

Determinations of circular dichroism spectra, thermal denaturation profiles, sedimentation coefficients, equilibrium molecular weights, DNA sizes, and histone contents were made by established methods. Mapping nuclease cutting sites in core particles labeled at the 5' ends with ^{32}P (using radioactive ATP and polynucleotide kinase) was carried out as previously described, except that DNA from poly(dCdC)·poly(dGdC) particles was treated with glyoxal before electrophoresis. Determinations of the torsional constraints induced on SV-40 DNA by association with histones were made by agarose gel electrophoresis after treatment with DNA topoisomerase I.

^{31}P-NMR spectra were obtained using a home-built spectrometer equipped with a Bruker 6.4 T superconducting magnet and coupled to a Nicolet 1180 computer. A number of spectra (5000-50000) were collected and Fourier transforms analyzed after signal averaging.

Major Findings: The kinetics of the chromatin core particle reassembly reaction in solution were quantitatively studied under conditions such that nucleohistone aggregation did not occur. Core particles, salt-jumped rapidly by dilution from 2.5 M NaCl (in which DNA and histones do not interact) to 0.6 M NaCl (in which core particles are nearly intact), reassemble in two distinct time ranges. Approximately 75% of the DNA refolds into core particle-like structures "instantaneously" as measured by several physical and chemical techniques with dead times in the seconds to minutes time range. The remaining DNA refolds with relaxation times ranging from 250 minutes at 0°C to 80 minutes at 37°C; this slow effect cannot be attributed to sample heterogeneity. The fraction of slowly refolding DNA and the slow relaxation time are independent of the core particle concentration. Transient intermediates present during the slow phase of refolding were identified as free

DNA and core particle-like structures containing excess histone. Mixing experiments with DNA, histones, and core particles showed that core particle-histone interactions are responsible for the slow kinetics of DNA refolding. Upon treatment of reassembling core particles with the protein crosslinking reagent, dimethylsuberimidate, the slow phase of the reassembly reaction was arrested and a 13 S particle containing DNA and two octamers of histone was isolated. Consistent with the nature of this kinetic intermediate, it is shown that in 0.6 M NaCl, core particles co-operatively bind at least one additional equivalent of histones with high affinity in the form of excess octamers. Also, core particles continue to adsorb considerably more histones with a weaker association constant of the order 10^5 M^{-1} (in units of octamers) to a maximum value of 12±2 equivalents (octamers) per core particle. The sedimentation coefficient increases with the two-thirds power of the molecular weight of the complex, as it would in the case of clustered spheres.

A reassembly mechanism consistent with the data has been defined, and other simple mechanisms excluded. In the proposed mechanism, core particles reassemble very rapidly and compete effectively with DNA for histones such that approximately one-third of the particles initially formed are complexed with an excess octamer of histones, and 25% of the total DNA remains uncomplexed. The amount of this unusual reaction intermediate decays slowly to an equilibrium value of about 10%, thereby leaving 9% of the total DNA uncomplexed. Approximate values have been calculated for the free energies, rate constants, and two of the activation energies which characterize this migrating octamer mechanism. This mechanism provides a means whereby histone octamers can be temporarily stripped off DNA at a modest free energy cost, approximately 2.6 kcal per nucleosome. Also, the properties of excess histone adsorption by chromatin and octamer migration suggest an efficient mechanism, consistent with observations by others, for nucleosome assembly in vivo during replication.

Reassembly of chromatin and nucleosomes at lower ionic strenghts, likely closer to those found in vivo, has been reported by others to require the participation of "assembly factors." We too find that direct mixing of the four smaller histones and 145 bp DNA at ionic strength 0.1 leads to a turbid mixture, containing large amounts of nonspecifically aggregated material. However, on incubation at 37° for 16 hours, the reaction mixture clears and about 70% of the DNA is found associated with histones in a complex which resembles, in a number of ways, the native chromatin core particle. Addition of larger excesses of histone, as expected from the results described above, leads to formation of aggregates, likely containing excess histone octamers bound to DNA and/or nucleosomes.

Similarly, we find that direct mixing in 0.1 or 0.2 M NaCl of SV-40 $DNAI^r$ with an equal mass of histones leads, after incubation for some hours at 37°, to induction of the torsional constraints characteristic of native SV-40 chromatin in a significant portion of the molecules. When the ratio of histone to DNA is increased to 2:1, used by others in attempts to reassociate at I=0.1, no indication of correct reassociation is obtained. Control

experiments suggest that this is again due to formation of aggregates; these can not be relaxed by topoisomerase.

The rate of the reassociation at physiologic ionic strength and the eventual degree of correct reassociation obtained can be markedly enhanced by inclusion in the reassociation mixture of polyglutamic acid (at twice the mass of histones). Within one hour of incubation at 37°, essentially all the SV-40 DNA is converted to a fully superhilical form, indicating faithful reassembly of the SV-40 minichromosome in vitro. The reassembly process is highly cooperative, in marked contrast to the mechanism observed during salt-gradient dialysis reassociation.

Histones alone in solution at these ionic strengths are present as a poorly-defined mixture of dimers and monomers, in contrast to the strictly-defined octamer which exists in the chromatin core particle. Our previous observations suggested that interaction with a negatively charged macromolecule (the DNA of an intact nucleosome) was sufficient to allow the histones to exist as an octamer; the suggestion from this work was that an anionic protein, exemplified by polyglutamic acid, might suffice to stabilize the histone octamer and, thereby, facilitate reconstruction of chromatin from histones and DNA at these lower ionic strengths. This suggestion has been confirmed; crosslinking a mixture of histones and polyglutamic acid at I=0.1 with dimethylsuberimidate lead to most of the histones being covalently tied together as octameric molecules.

These studies demonstrate that histone octamers can interact with negatively charged proteins in a stable form. As such, they provide reasonable mechanisms for a number of puzzling observations in the older chromatin literature; these include the stimulatory effects of acidic proteins on both transcription and replication; the possible role of acidic proteins, in particular phosphorylated nonhistones, in gene regulation; the association of highly charged anionic proteins with putatively active chromatin; and the presence of large quantities of an acidic protein in the egg of amphibians, a cell which contains a store of histones sufficient for a number of cell divisions. Acidic proteins are envisioned as providing a binding "sink" for the excess histones, or those transiently dissociated during transcription, thereby facilitating removal of histones from DNA during the process of RNA synthesis.

We have made a major effort in the past year towards defining the types of DNA sequences which might preclude folding of the nucleic acid into a typical nucleosome conformation; if such a process occurred in vivo, it would provide an attractive mechanism for localized phasing of core particle structures in the genome, and, thereby, a possible structural element involved in facilitation of readout of the genetic message. In these studies, we have used two approaches. The first, association of histones with SV-40 DNA to look directly for selective association or nonassociation of the basic proteins with specific DNA sequences, is summarized in a related report (15101-08). The second approach, widely used in studies of DNA structure but only rarely applied to protein-DNA interactions, is to amplify the possible structural effects of DNA sequence through the use of synthetic polydeoxyribonucleotides.

Thus, we have attempted to form semi-synthetic chromatins (and therefrom, core particles) from the two complementary homopolymers, poly(dG)poly(dC) and poly(dA)poly(dT) and the two alternating copolymers, poly(dAdT)poly(dAdT) and poly(dGdC)poly(dGdC). As an added potential benefit of such studies, we felt that, should any of the synthetic polydeoxyribonucleotides form core particles, the structural features of the resultant nucleoprotein should be significantly better defined than those of native particles containing random-sequence DNA, due to the lack of sequence heterogeniety and its effects on physical and chemical features of the particle in the semi-synthetic chromatin.

We have used four criteria, characteristic of native chromatin, to judge whether the synthetic DNA molecules could be folded by the histone octamer into a chromatin-like structure: 1) digestion by DNAase I to yield single-stranded fragments which are multiples of 10 bases; 2) digestion by micrococcal nuclease to yield double-stranded fragments which include a 140 base pair core particle segment; 3) induction of a biphasic melting profile for the DNA and 4) generation of a typical beaded string morphology for the complex. Judged by all four criteria, the complementary homopolymers failed to form chromatin-like structures. We envision the reasons for this as follows. Poly(dG)poly(dC) tends to form A-form DNA structures at high ionic strengths (low relative humidities), such as those used for the association. Inspection of the surface topography of the A-form of DNA suggests that the disposition of the phosphodiester backbone may differ sufficiently for this conformation vs. B-form DNA to disallow matching of the required electrostatic interactions between DNA and the histone octamer core of the nucleosome; thereby folding of the DNA is precluded. Poly(dA)poly(dT), in solutions of high ionic strength, disproportionates to form a single strand of poly(dA) and an A-form triple helical structure, poly(dA)poly(dT)$_2$. Again this A-form DNA is likely incapable of folding around the histone octamer to form a proper nucleosome. Since both RNA-DNA hybrids and triple-stranded RNA-DNA$_2$ complexes are A-form structures, this result implies a plausible mechanism for transient dissociation of histones from DNA being transcribed, extending the octamer transfer mechanism detailed above and previously.

In contrast, the alternating copolymers form chromatin and core particles quite equivalent to those formed with native, random sequence DNA. One major difference between these complexes and native particles is notable; that is that due to the lack of sequence heterogeniety in the semi-synthetic chromatins, the properties of the core particles are almost totally dependent on histone-DNA interactions, uninfluenced by the sequence-variable properties of the native nucleic acid. This leads to an order of magnitude improvement in the resolution of the structural features of the core particles containing synthetic DNA as opposed to those containing random sequence native DNA. For example: we have been able to define the relative cutting site susceptibilities to DNAse I for the core particle at a level of single base resolution (showing that all sites in the DNA can be cut, sites at multiples of about 10 are preferential but not exclusive sites); accurately define the distance between preferential sites as 10.4-10.5 bases, a value consistent with the helical repeat of B-form DNA in solution; demonstrate DNA sequence dependent differences in the pattern of nuclease cleavage of the core particle (presumably reflecting sequence dependent differences in core particle structure);

confirm the suggestion of others that the organization of DNA in the core particle involves close side-to-side packing of the DNA helices into a form resembling PSI-form DNA; more accurately demarcate the sites of interaction of various histone amino terminal regions with core particle DNA and the overall symmetry of the organization of DNA in the chromatin core particle; and accurately define the mechanism of a reversible conformational transition of the chromatin core particle which may be relevant to the function of chromatin in transcription in vivo. The results which we have obtained thus far with the semi-synthetic chromatins preclude several published proposals for explanation of features of core particle structure, confirm others, and place rather severe constraints on future models for the organization of chromatin.

As one example to illustrate the potential of the use of semisynthetic chromatin for study of structure, we will describe the results of a detailed analysis of the thermal denaturation of core particles constructed by association of the inner histones and poly(dAdT)poly(dAdT). The well separated melting transitions (35° for the first and ca. 68° for the second) observed on heating synthetic core particles containing poly(dAdT)·poly(dAdT) have allowed physicochemical studies to define the mechanism of the first, reversible transition. The shape of the particle, assessed by sedimentation velocity measurements, becomes extended at temperatures about 10° below the optical melting temperature; this change is unaffected by cross-linking the histones into a stable octamer. A portion of the DNA undergoes a transition to a structure more like that of protein-free poly(dAdT)·poly(dAdT), as judged by circular dichroism measurements, also at temperatures below those necessary for optical melting. Digestion of 5'-^{32}P-labeled particles with S1 nuclease at a temperature partly through the first optical transition demonstrates that the initial phase in melting core particles is separation of about 20 base pairs of DNA at each end of the particle segment from the histone core.

Others have proposed that transcription of chromatin DNA associated with histones might involve dissociation of the core particle into two symmetrical half-nucleosomes, each consisting of a segment of DNA associated with a heterotypic tetramer of histones. The current results clearly do not provide evidence for such a conformational transition on gentle heating of core particles. Rather, to the extent that in vitro studies reflect or place constraints on those structural transitions which might occur in vivo, they suggest that an unwinding of DNA from the end of the core particle might be the primary event in generation of a transcriptionally active nucleosome. Possible mechanisms which might facilitate this are considered elsewhere in this report.

In collaboration with H. Shindo (LCP, NIAMDD), we have investigated the conformation of DNA in the semi-synthetic core particles and the same 145 base pair poly(dAdT)poly(dAdT) when protein-free using ^{31}P-NMR. In contrast to the spectrum of native random-sequence DNA of the same size (a broad envelope), the spectrum of the alternating copolymer shows two discrete peaks, separated by 24 Hz at 109.3 MHz. The peaks are of equal intensity at all temperatures below the T_m; as the DNA melts, the peaks coalesce into

a single sharp signal indicating equivalent environments for all the phosphorus atoms in the randomly structured, denatured DNA. Detailed analyses of the possible basis for the two ^{31}P resonances leads to the conclusion that the phosphodiester backbone of this DNA contains two distinct phosphorus environments, likely in an alternating array. Previous studies of the NMR properties of the copolymer have failed to detect this feature; large DNA sizes have led to broad line widths, obscuring the split signals. This is the first indication of variations in the structure of the phosphodiester backbone of DNA related to base sequence; we feel it likely that this may be a general phenomenon for all DNA's.

At 21°C, the poly(dAdT)poly(dAdT) core particle ^{31}P-NMR spectrum is fit well by a single Lorenzian distribution with a line width of 110 Hz. This signal is significantly broader than that for the 145 base pair poly(dAdT)·poly(dAdT) alone; as noted above, the latter consists of two resonances, approximately equal in intensity, with average line width 41 Hz. Major changes in the spectrum ensue on heating the core particle preparation. At 40°C, a temperature below that necessary for strand separation in the first, reversible thermal transition of the particle, the NMR spectrum is asymmetric; curve analysis as a two component spectrum suggests that a distinct signal corresponding to 15 -20% of the phosphorus atoms now appears. At 50°C, about 30% of the total signal area is a markedly narrow resonance separated from the broad component; at this temperature, the first optical transition is nearly complete. Finally, above the second melting temperature for the particle, all the phosphorus resonances are a single, narrow line. In conjunction with other results, these data suggest four states for the core particle at increasing temperatures. Additionally, analysis of the spectrum of the unmelted core particle and its differences from protein-free DNA of the same length suggests that the conformation of the phosphodiester backbone and/or its interactions with histones along the length of the core particle DNA segment may not be uniform.

Significance to Biomedical Research and the Program of the Institute: Elucidation of the structure of the eukaryotic nucleoprotein complex, chromatin, is necessary for understanding the mechanism of gene regulation in higher organisms. Our current studies aim at complete understanding of the structural relationships between histones and DNA to create the nucleosome, the basic structural element of chromatin, and higher orders of chromatin structure.

Proposed Course of Project: Investigations of histone-DNA interactions and the mechanisms of folding of DNA by interaction with the histone octamer will continue; particular emphasis will be placed on definition of the differences between core particles containing different sequences of DNA (e.g., poly(dAdT) vs. poly(dGdC). We will extend our studies of structure to a higher level of DNA organization, attempting to define the structure of particles containing 8-16 nucleosomes by methods analogous to those we have successfully employed on smaller particles. Particular emphasis will be placed on attempts to recreate in vitro the proper structure of large chromatin segments using polyglutamic acid as a tool in reassociation of histones and DNA; this is a mandatory prerequisite to experiments using cloned gene sequences to attempt to reconstruct a functional gene as chromatin in vitro.

Publications:

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Simpson, R.T., Whitlock, J.P. Jr., Bina-Stein, M., and Stein, A.: Histone-DNA interactions in chromatin core particles. Cold Spring Harbor Symp. Quant. Biol. 42: 127-136, 1978.

Simpson, R.T. and Künzler, P.: Chromatin and core particles formed from the inner histones and synthetic polydeoxyribonucleotides of defined sequence. Nucleic Acids Res. 6: 1387-1415, 1979.

Tack, L.O. and Simpson, R.T.: Location of histone lysyl residues modified by in vitro acetylation of chromatin. Biochemistry (in press).

Stein, A.: DNA folding by histones: the kinetics of chromatin core particle reassembly and the interactions of nucleosomes with histones. J. Mol. Biol. 130: 103-134, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 15101-08 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Analysis of the Active and Inactive Portions of the Eukaryotic Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert T. Simpson	Medical Director (R) (T)	LNE NIAMDD
Others:	Donald J. Gruol	Senior Staff Fellow	LNE NIAMDD
	Grant A. Bitter	Staff Fellow	LNE NIAMDD
	Mark O. J. Olson	Associate Professor	Department of Pharmacology, Baylor College of Medicine

COOPERATING UNITS (if any)
Laboratory of Physical Biology, NIAMDD

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.25	2.75	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Effects of histone modifications on structure of higher order chromatin have been studied; H1 phosphorylation apparently loosens the structure somewhat, this is reflected in melting temperature and sedimentation behavior. Salt gradient dialysis experiments have shown that nucleosome placement can occur at any site on SV-40 DNA; no sequence can not be folded by histones. Micrococcal nuclease and DNAase I both excise the receptor for triiodothyronine (T3) from chromatin early in digestion as a 6S sedimenting species. With higher digestion, the sedimentation coefficient decreases to 5S, still greater than the free receptor at 3.5S. We suggest that the receptor is associated with DNA and an extended nucleoprotein in the 6S primary product. Yeast RNA polymerase II has been isolated and purified to >80% purity. The enzyme transcribes core particles at a rate half that of their constituent protein-free DNA. Surprisingly, about 4 polymerase molecules can bind to either core particles or their DNA and crosslinked core particles are transcribed as readily as native nucleosomes.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To investigate the physical and chemical differences between portions of the eukaryotic genome which are transcribed and those which are repressed. To determine the features necessary and sufficient for correct transcription of animal cell chromatin and isolated DNA in vitro.

Methods Employed: HeLa and HTC cells are grown under standard conditions and in some cases, incubated with sodium n-butyrate, zinc chloride, or cycloheximide. Nuclear preparations, digestions with nucleases, and separation of chromatin particles are done by methods standard in this laboratory. DNA fragments are analyzed by gel electrophoresis under native and denaturing conditions and proteins are separated by gel electrophoresis in the presence of sodium dodecyl sulfate.

Histones are isolated from chicken erythrocyte chromatin by salt extraction and purified by gel filtration. Association of histones with SV-40 DNA (either form I or I^{-}) is performed by salt gradient dialysis. Associated samples are treated with appropriate restriction endonucleases or DNA topoisomerase I and the torsional constraints induced on the DNA and the fraction of linear and circular molecules determined by agarose gel electrophoresis.

Circular dichroism spectra of chromatin particles are determined in a Cary Model 61 spectropolarimeter. Analytical ultracentrifugation is performed in Beckman Model E instrument equipped with RTIC, ultraviolet scanner optics and multiplexer. Thermal denaturation analyses are performed in an ACTA III spectrophotometer equipped with sample changer, temperature programmer, and platinum resistance thermometer.

^{125}I-triiodothyronine (T3) was incubated with chromatin or chromatin fractions as previously detailed. Assessment of binding was by either 1) the hydroxyapatite method previously described or 2) a method using small columns of Sephacryl S-200 (which binds T3 tightly under the assay conditions) to separate free from bound hormone.

Target size analysis of chromatin samples is done following irradiation of frozen samples by a high energy (11 MEV) beam of electrons in a linear accelerator. Binding of labeled T3 is determined as a function of irradiation dose and approximate molecular sizes calculated by standard methods from semilog plots of the loss of binding capacity.

Nuclei are isolated from spheroplasts prepared by zymolyase treatment of yeast grown to mid-log phase in a synthetic medium. RNA polymerase II is isolated from yeast by polymin P precipitation, ammonium sulfate precipitation, and chromatography on DEAE-cellulose, DEAE-Sephadex, and phospho-cellulose. Enzyme assays are conducted in standard fashion, using ^{3}H-UTP and DNA or chromatin particles as substrates. Determination of numbers of polymerase binding sites are carried out using heparin sulfate, ammonium sulfate, or Rifampicin A103 to inhibit reinitiation.

Major Findings: We have previously studied the effect of histone acetylation on structure of chromatin at the nuclear and isolated core particle levels. We have now extended these studies, evaluating the effects of histone acetylation induced by treatment of HeLa cells with sodium n-butyrate on the structure of higher order chromatin preparations containing either 14-18 nucleosomes or 6-10 nucleosomes. Parallel experiments have examined the effect of hypo- and hyperphosphorylation of histone H1 on such chromatin isolated from HTC cells treated with cycloheximide or zinc chloride, respectively.

Chromatin segments (putatively the "superbeads" of Renz) are excised more rapidly from nuclei containing hyperphosphorylated H1 than from controls or those with hypophosphorylated H1. At any given time in the digestion, the size of the chromatin from the hyperphosphorylated nuclei is smaller. Over an ionic strength from 0.1-0.5 M, the sedimentation behavior of chromatin containing modified H1 or acetylated H3 and H4 are experimentally indistinguishable from their appropriate contols; the modifications do not seem to affect the loosening of particle structure that accompanies the salt-induced dissociation of H1 histone. In contrast, the sedimentation coefficient of chromatin containing hyperphosphorylated H1 does not decrease on lowering the ionic strength from 0.1 M to 10 mM, suggesting that this chromatin is already in a relatively extended form at physiological ionic strength, contrasted to the compact form of the material containing less modified histone. This is supported by the observation that, for equivalent DNA sizes, the sedimentation coefficient of chromatin with phosphorylated H1 is always lower (at I = 0.1 M) than that for control samples. Both phosphorylation and acetylation lead to small but reproducible decreases in the melting temperature for the chromatin samples, again suggesting a slight destabilizing effect of the charge altering chemical modifications of the histones on the structure of the nucleoprotein. These observations are less dramatic differences than we expected if the chemical modifications produced their effects on chromatin function at this level of structure.

In vivo, chromatin containing H1 appears to be packed tightly into (minimally) 100 Å diameter fibrils or (more likely) 250-300 Å diameter supercoils or arrays of superbeads. This would likely create a formidable problem for reading of DNA sequences by regulatory proteins involved in modulation of genetic activity. An attractive mechanism to make regulatory DNA sequences available for reading involves a localized phasing of nucleosome structures on DNA; if a portion of the DNA was not wrapped around a histone octamer, it and certain of its flanking sequences might be available for interaction with other proteins. Varshavsky has recently shown that the native SV-40 minichromosome has a region of the DNA which is always available for cleavage by single or multiple cut restriction endonucleases and DNAase I. This region includes the origin of replication and both promoters, providing support for the hypothesis outlined above. We have begun testing the hypothesis by reassociating histones with SV-40 DNA in vitro and examining the susceptibility of the resultant complex to cutting by three single cut restriction endonucleases, Bgl I, which cuts in the origin region (cuts 100% of the native minichromosomes), and Eco RI and Bam HI, which cut elsewhere (cut about 30% of the molecules in the native state).

Thus far the results obtained have failed to support the hypothesis outlined above. Thus, complexes formed with the four smaller histones (H2A, H2B, H3 and H4) from either chicken erythrocytes or butyrate-treated HeLa cells and either form I or form I^r SV-40 DNA have shown random digestion by all three nucleases under a variety of ionic conditions and temperatures. In these cases it thus appears that the possibility of nucleosome formation is not dictated by DNA sequence. We will extend these studies to include H1 histone, using the polyglutamic acid method of reassociation described in a related report (15100-09). If this too fails to generate phasing, we will conclude that either 1) phasing is a consequence of events occurring at the time of replication of the chromosome or 2) other proteins which are known to bind near the origin may preclude nucleosome formation. For the latter case, T antigen is known to bind to SV-40 DNA in this area and the possibility can be tested.

Another method for assessing the interactions of histones, DNA and other proteins in the region of regulatory sites in chromatin derives from our studies of the interactions of the nucleoprotein with the thyroid hormone, triidothyronine (T3). T3 is likely involved in regulation of gene expression in certain tissues at the level of transcription. We have previously described extensive studies of the interaction of T3 with chromatin from several rodent tissues using an assay method superior to those previously published by others. The binding is of high affinity (K_d = 2 x 10^{-11} M) to a limited number of sites. We are now dissecting chromatin by nuclease digestion and examining the size distribution of binding sites; this should allow definition of the type of chromatin array in the neighborhood of the binding protein and its associated DNA.

Early in digestion of nuclei with micrococcal nuclease, a binding peak appears on sucrose gradients at 6S. No evidence has been obtained for association of the receptor with monomer or higher-order nucleosomes (up to tetramer). With increasing digestion the binding peak slows to sediment at 5S, still well above the sedimentation coefficient of the salt-dissociated receptor, 3.5S; by analogy with the trimming of nucleosomes during formation of core particles from chromatin, this can be interpreted to indicate that the 6S species contains DNA in addition to the T3 receptor (and possibly other proteins). We feel it is quite possible that the 6S species is an extended nucleosome, like that shown to occur for the ribosomal genes in chromatin, although proof of this hypothesis remains to be offered. Digestion of nuclear chromatin with DNAse I, an enzyme which preferentially degrades active genes, also leads to a 6S T3 binding fragment early in digestion. However, in this case a 12-13S binding peak is also observed early and lost at later stages of digestion. The relationship of these observations to those obtained with micrococcal nuclease remains to be elucidated; it may be related to the preferential sites for cutting of the two nucleases - micrococcal nuclease in the linker region and DNAase I within the nucleosome.

The higher sedimentation coefficient of the digested T3 binding peak relative to the free receptor suggests that the receptor is tightly bound to other chromatin components. Direct measurement of the size of the complex in

native chromatin would require an enormous purification, likely not feasible now. We have approached the question of the size of the T3 receptor in intact and variously degraded chromatin in collaboration with E. Kempner (LPB, NIAMDD) using high energy electron irradiation and target analysis theory. Target sizes for the salt-dissociated receptor are consistent with its estimated molecular weight from other studies of about 50,000. Surprisingly, the isolated 6S peak from micrococcal nuclease digests of chromatin had a target size much larger than that for either the salt-dissociated receptor or the receptor when in sonicated chromatin. Resolution of these apparent inconsistencies will require not only further study of the T3 system but also model system studies to determine the effect of noncovalent complexing of proteins to high molecular weight DNA on the apparent target size for the smaller protein molecule.

An alternative approach to defining the structure and composition of transcriptionally active chromatin involves reconstruction of the transcribing system with defined DNA sequences, appropriate eukaryotic polymerases, and various proteins. We have begun this approach in the past year. Thus far, we have isolated RNA polymerase II from yeast and purified the enzyme to over 80% homogeniety (free of contaminating enzymatic activities) and begun to investigate the interactions of the polymerase with defined nucleoprotein substrates.

Yeast RNA polymerase II transcribes both 145 base pair DNA and chicken erythrocyte core particles with facility; the rate of transcription of DNA, under conditions where reinitiation can occur and enzyme is in excess over DNA, is about double that of the core particles. The number of polymerase molecules bound per molecule of DNA is equal for naked DNA and core particles; surprisingly it is high, 3-5 molecules of enzyme per molecule of DNA. This value is about 20 times higher than that observed for prokaryotic RNA polymerase binding to high molecular weight DNA and 200 times higher than the value for high molecular weight chromatin. Core particles with acetylated histones are transcribed at equivalent rates to those with largely unmodified proteins. Somewhat surprisingly, core particles in which the histones have been covalently cross-linked into an octamer with dimethylsuberimidate are also transcribed at equivalent rates to native particles. This would suggest that dissociation of the histone octamer into halves, proposed by others as a necessary feature of transcription of histone-bound DNA, does not occur in vitro; a caveat is that we do not yet know the length of RNA produced by transcription of isolated core particles by RNA polymerase II in vitro. The high level of binding of RNA polymerase to isolated core particles demonstrates that association of DNA with a histone octamer apparently has little effect on the availability of the nucleic acid to interact with large proteins, a conclusion recently made for small molecules by others. It appears that regulation of transcription in eukaryotic cells must lie either in the interactions of repressive regulatory proteins with DNA near promoter regions of the nucleic acid, or be based in higher order organization of chromatin, perhaps involving the participation of histone H1 in supercoiled or beaded structures.

Significance to Biomedical Research and the Program of the Institute:
One of the most direct approaches to understanding the basis for transcriptional

regulation is comparison of the composition and structure of transcribed with repressed segments of chromatin. This separation has proven to be difficult if not impossible with current tools and unique genes. We therefore, have concentrated on probing the structure of active chromatin regions with a series of small molecules, hormones, nucleases, and polymerases. Progress in these areas will aid in achievement of our eventual goal, description of the mechanism of transcriptional regulation in animal cells and thereby the features responsible for the stable differentiated state of a normal mature cell. Understanding of these features will facilitate understanding of neoplastic transformation and normal development and differentiation.

Proposed Course of Project: We will attempt to determine proper conditions for transcription of unique genes in vitro, both as protein-free DNA (e.g., cloned globin gene or yeast genes inserted in the 2um plasmid) and eventually as native or reconstituted chromatin. Using one of two unique biological systems we will attempt the isolation of an active gene in the form of its native nucleoprotein complex, chromatin. The section of the project related to T3 interactions with chromatin is terminated as an N.I.H. project; some aspects of this work will be continued by Dr. Donald Gruol at the Salk Institute.

Publications:

Bustin, M., Kurth, P.D., Moudrianakis, E., Goldblatt, D., Sperling, R., and Rizzo, W.: Immunological probes for chromatin structure. Cold Spring Harbor Symp. Quant. Biol. 42: 379-388, 1978.

Simpson, R.T.: Structure of the chromatosome, a chromatin particle containing 160 base pairs of DNA and all the histones. Biochemistry 17: 5524-5531, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZOI AM 15102-19 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Study of Ribonuclease (Barnase) and its Inhibitor (Barstar) from Bacillus amyloliquefaciens.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert W. Hartley Research Physicist LNE NIAMDD

Other: None

COOPERATING UNITS (if any) Mr. D.L. Rogerson, Large Scale Laboratory, LNE/NIAMDD/NIH
Collaboration: D.M.Blow, Dept.Biophys., Imperial College, London;
Chothia, Dept.Chem., University College, London;
G.C. Dodson, Dept. Chem., Univ. of York; and R.S. Sheppard, Lab. of Molecular Biology, MRC, Cambridge.

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two proteins, barnase, the extracellular ribonuclease of Bacillus amyloliquefaciens, and barstar, its intracellular inhibitor, are being developed as a model system for the study of protein folding and protein-protein interactions. Amino acid sequence determination and X-ray structure characterization are being carried out along with thermodynamic studies of folding reactions. A peptide complementation system for barnase has been developed which will be used to investigate the roles of specific residues in both folding and enzymatic activity.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To further characterize and investigate this enzyme and its natural inhibitor by physical, chemical, and biological techniques. To develop methods of culture, genetic manipulation and genetic analysis so that the organism and its product enzyme-inhibitor pair may be applied to a broad study of structure, function and synthesis.

Specific objectives are: 1) the determination of the amino acid sequence and three-dimensional structure (by X-ray diffraction) of both proteins and the structure of their complex; 2) thermodynamic and kinetic analysis of structural transitions produced in both proteins by heat or denaturing reagents; 3) analysis of the folding and activity of both by means of peptide complementation systems analogous to those being used for the study of pancreatic ribonuclease and micrococcal nuclease; 4) all of the above with chemically and/or genetically modified sequences.

Methods Employed: B. amyloliquefaciens (strain H and derivatives and several others) are grown in synthetic media. Barnase is adsorbed from the culture medium onto phosphocellulose, barstar is extracted from the cells. Their purifications make use of ion exchange, gel-permeation and affinity chromatography.

Work on the determination of amino-acid sequences involves amino acid analysis, enzymatic digestions, peptide separations and peptide sequencing by dansyl-Edman and difference-Edman techniques. Peptide separation and amino acid analysis are by automated ion-exchange chromatography using a modified JEOL analyzer equipped for detection with Roth's orthophthaldehyde reagent. Dansyl amino acids are identified by thin-layer chromatography on polyamide layers.

The reversible transitions undergone by barnase and barstar and their derivatives under the influence of temperature changes or denaturing agents are studied primarily by ultraviolet and fluorescence spectroscopy, but also by optical rotatory dispersion, circular dichroism, exclusion chromatography with the use of proteolytic enzymes and by equilibrium binding studies. These techniques are also used to study complementation reactions between natural and synthetic peptide sequences of the two proteins as well as conformational properties of each peptide alone. Studies of the peptide derivatives also include use of antibodies, chromatography, crystal growth, etc.

Crystals are grown in hanging drops (10 µl) suspended over suitable solvents. Isomorphous crystals containing heavy atoms are prepared by soaking crystals in solutions of appropriate heavy atom compounds. The three dimensional structure of barnase is being determined by methods of X-ray crystallography.

The Merrifield procedure has been used to synthesize portions of the barnase sequence.

Major Findings: The sequence of barnase, along with those of several homologous bacterial enzymes, have been shown to be homologous to a group of extracellular fungal ribonucleases.

Significance to Bio-Medical Research and the Program of the Institute: The small size, stability, easily measured enzyme activity and absence of disulfide bridges in barnase make it an excellent choice for a long-range study of the relation between the sequence of an enzyme and its structure and function. The inhibitor is an even simpler protein and should be useful in a similar fashion. Together, they offer a model system for the study of protein-protein interaction.

The nature and extent of the thermal transition of barnase gives strong support to the concept that the three-dimensional structure of a protein may be completely determined by its amino acid sequence. The simplicity and all-or-none nature of the transition should make it useful for thermodynamic analysis is the factors involved in maintaining a folded protein structure. The production of both of these proteins by the same organism is also of biological interest.

The discovery of homology between prokaryotic and eukaryotic ribonucleases greatly extends the significance of this work. Study of the whole group of enzymes should help our understanding of evolutionary relationships on the broadest scale. Comparisons of their sequences and structures may contribute substantially to the solution of the sequence-directed-folding problem.

Proposed Course of the Project: Collaboration with the authors in England on the X-ray structure and on peptide complementation will continue.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 15104-07 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on Eukaryotic DNAs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George Rushizky	Research Chemist	LNE	NIAMDD
Other:	Krishna Baksi	Visiting Fellow	LNE	NIAMDD

COOPERATING UNITS (if any)

Pilot Plant, LNE, NIAMDD

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SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	none

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Restriction endonucleases from various microorganisms were studied, and one such enzyme (Pal I) purified to homogeneity. Work is in progress on the fractionation and isolation of large DNA fragments with biological activity, such as the origin(s) of replication of Vaccinia virus.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To isolate specific fragments from large DNAs, labeled or in amounts sufficient for studies of their structure and biological function. Emphasis is placed on improvement of existing methods or development of new ones.

Methods Employed: DNAs were prepared from phages and vaccinia virus grown by standard procedures in bacteria and HeLa cells, respectively. The DNAs were hydrolyzed with restriction enzymes isolated in the lab or available commercially, and the products characterized by various procedures. Type II restriction endonucleases were purified from the cells of different microorganisms.

Major Findings: The restriction endonuclease Pal I was purified 1650-fold with a yield of 33%, mostly by affinity chromatography. The enzyme was free of contaminating nucleases as determined by ligation and recutting of Pal I digests of DNA, and homogeneous as a protein as judged by polyacrylamide-SDS electrophoresis using 15-30 µg of enzyme. Physical and catalytic properties of the enzyme were examined. A paper on the purification of this enzyme has been accepted for publication.

A survey of various bacteria for restriction endonucleases with new specificities was undertaken using affinity chromatography methods as employed for Pal I purification. No new enzymes were found. Isoschizomers of known 4-base specific enzymes were found in such hosts as Arthrobacter polychromogenes and Serratia marcescens.

Fractionation of large DNA fragments on base-specific absorbents has been examined. Useful adsorbents were poly rC-Sephadex and phenyl neutral red polyacrylamide gel. Both bind (G,C) base pair lengths of 6 or more. Restriction fragments near the origin of replication of SV 40 DNA contain such G-C rich segments and are selectively bound to these adsorbents. While other DNAs may not contain such sequences near their origin of replication, the adsorbents provide a useful addition to current methods for the fractionation of restriction endonuclease digests of DNAs. This project is not completed.

Work is in progress on the determination of the site of the origin(s) of DNA replication of vaccinia virus. This involves pulse-labeling of HeLa cells for several time intervals after infection with a high multiplicity of virus to provide synchrony, isolation of full-length DNA by sucrose gradient centrifugation, restriction endonuclease hydrolysis and examination of Dintzis-type labeling patterns.

Significance to Biomedical Research and the Program of the Institute: The procedure for the purification of Pal I as well as the enzyme itself may prove useful for studies on the structure and function of type II restriction endonucleases.

Restriction endonuclease digests of vaccinia DNA represent an excellent source of defined DNA fragments of limited number, with biological activity and high molecular weights such as 10-33 x 10^6, suitable for the development of fractionation procedures.

Assignment of the origin(s) of replication of vaccinia DNA would enable a number of experiments. For example, current research on SV 40 DNA synthesis indicates that early transcription starts at the origin of replication on a chromosomal form of DNA. Elements which control the initiation of both DNA and RNA synthesis may therefore be localized in this region and may correspond to those factors which control the arrangements of nucleoproteins at that site. It is assumed that vaccinia virus may be under similar controls as SV 40.

Proposed Course of the Project: Goals include the determination of the origin(s) of DNA replication of vaccinia virus as the Hind III fragment(s), and further hydrolysis with other restriction endonucleases to a size suitable for nucleotide sequence determination. This involves the elucidation of other restriction endonuclease maps than the one known at present, and additional methods for the fractionation of large DNA fragments.

Publications

Baksi, K., Rogerson, D.L., and Rushizky, G.W.: Rapid, Single-Step Purification of Restriction Endonucleases on Cibacron Blue F3GA. Biochemistry 17, 4136-4139, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZOI AM 15105-03 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
A Developmental Transition in Mosses as a Model for the Study of Eukaryotic Genome Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert W. Hartley Research Physicist LNE/NIAMDD

Other: None

COOPERATING UNITS (if any)
Mr. D.L. Rogerson, Jr., Large-scale Laboratory, Laboratory of Nutrition and Endocrinology, NIAMDD
Office of the Chief, Laboratory of Nutrition and Endocrinology, NIAMDD

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Laboratory of Nutrition and Endocrinology

SECTION
Developmental Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	0.8	0.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The developmental transition of the moss gametophyte from filamentous protonema to leafy gametophore is being developed as a model for eukaryotic systems in which large blocks of genes are switched on or off. The transition (in Nanomitrium austinii) may be induced by cytokinins or, under certain conditions and reversibly, by changes in temperature.

The coordinate control of the two structural genes of ribulose bis-phosphate carboxylase is also being investigated in these organisms.

PHS-6040
(Rev. 10-76)

Product Description

Objectives: In their development, mosses pass through two distinct generations, represented by the haploid gametophyte and the diploid sporophyte. The haploid generation is further divided into at least two distinct morphological stages, the mature "leafy" gametophore being preceded by the purely filamentous protonema. The transition between these two forms may, in some cases, be controlled by temperature or by plant growth substances of the cytokinin (6-N substituted aminopurine) type. The current objective is to bring into aseptic culture one or several moss species and study this discrete transition as an instance of coordinated gene-switching in a typical eukaryote. The system will also be used to study the enzyme ribulose bis-phosphate (RuBP) carboxylase. This enzyme (by a large factor the most abundant protein on earth) is of great practical importance to agriculture, but interest in the present context is in its mode of synthesis. Its two different subunits are coded in different genomes and synthesized in different cellular compartments, presenting a pretty problem in coordinated control of genetic information.

Methods Employed: Aseptic cultures are obtained by spreading the contents of surface-sterilized spore capsules onto suitable agar media. Culture thereafter is largely by standard microbiological techniques.

Protonemal tissue is obtained by growing it on a semipermeable membrane (overlying agar) from which it can be scraped, or by deep liquid culture in volumes up to 10 L. Large numbers of individuals are obtained by chopping up protonemal tissue in a Waring blender. Protonemal fragments are mutagenized with MNNG and a variety of mutant searching techniques carried out.

Protoplasts (osmotically fragile cells lacking any cell well) are obtained by treatment with a commercial cellulase preparation (Driselase) obtained from the basidiomycete Irpex lacteus.

Proteins are extracted from protonema or from lysed protoplasts and separated and studied by various chromatographic and/or electrophoretic techniques.

The RuBP-carboxylase is assayed by a method involving the fixation of C-14 labelled CO_2.

Major Findings: It has been found that N. austinii is almost an obligate phototroph. That is, it grows only very slowly in the absence of either light or CO_2 even when supplied with a wide variety of energy and carbon sources (sugars, amino acids, etc). It is this characteristic which has defeated efforts at auxotroph selection by the use of photodynamic inhibitors. Since the end products of photosynthesis are in fact sugars, it seems likely that the difficulty is in getting external sugars into the cells.

Soluble proteins may be extracted from osmotically lysed protoplasts or, more simply, from whole protonemal tissue in the presence of 1% Triton to destroy cell membranes. More than 50% of the stainable protein in either of

these extracts (on SDS gel electrophoresis) is made up of the large and small subunits of RuBP carboxylase. The enzyme is readily purified by two chromatographic steps, and the pure (denatured) subunits may be obtained therefrom by gel filtration in SDS. It appears to be similar in size and subunit structure to its homologs in both algae and angiosperms.

Significance to Biomedical Research and the Program of the Institute: The moss protonema-gametophore transition offers a convenient system for approaching the general problem of gene control in eukaryotes, particularly in the developmental aspect where large sections of the genome are turned on or off.

The RuBP carboxylase system offers access to another gene-control system in a typical eukaryote amenable to microbiological techniques.

Proposed Course of Project: Development of genetic background information with which to study developmental mutations and the search for such mutations will continue.

Identification and isolation of proteins unique to the particular developmental states will be attempted.

The biochemical fate of the effective cytokinins will be investigated, including a search for specific receptors.

The RuBP carboxylase will be further characterized and methods for obtaining mutations in the genes involved in its synthesis investigated.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 15200-19 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on Folic Acid (Dihydrofolate Reductase)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Bernard T. Kaufman	Research Chemist	LNE NIAMDD
	Dr. Elizabeth Barbehenn	Staff Fellow	LNE NIAMDD

COOPERATING UNITS (if any)
Dr. James H. Freisheim, Department of Biological Chemistry, University of Cincinnati, College of Medicine, Cincinnati, Ohio

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SECTION
Vitam Metabolism Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Dihydrofolate reductase continues to be of considerable pharmacological, biochemical and now genetic interest because this enzyme appears to be the site of action of the powerful and widely used chemotherapeutic drug, methotrexate. Since many of the unusual properties of the animal enzymes appear to be related to a unique sulfhydryl group, our current studies are concerned with the structure (amino acid sequence) of the chicken and bovine liver enzymes and the reactivity of these sulfhydryl groups. Thus sequence studies revealed that in both enzymes the single -SH group is located in the N-terminal region of the protein. Treatment of the chicken liver enzyme with an excess of sodium tetrathionate not only resulted in the covalent attachment of one mole of thiosulfate to this single -SH group but also resulted in a 5 to 10-fold increase in activity over the native enzyme. This is the first case of an enzyme exhibiting activation rather than destruction upon treatment with this reagent. Although the beef liver enzyme is activated by organic mercurials as well as high concentrations of urea, no reaction is observed with tetrathionate.

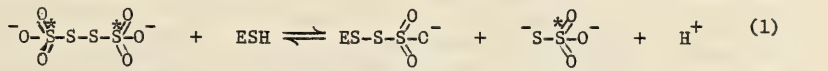
PHS-6040
(Rev. 10-76)

Project Description

Objectives: The interaction of drugs, substrates and cofactors with dihydrofolate reductase continues to be of considerable pharmacological and biochemical interest since this enzyme appears to be the site of activity of the powerful and useful chemotherapeutic agent, methotrexate. Dihydrofolate reductase which is concerned with the conversion of the essential vitamin, folic acid, to the biologically active cofactor, tetrahydrofolate, has a central role in cellular proliferation. For this reason we have continued to study the isolation, purification and properties of the reductases from various sources, particularly animal livers. Although the enzymes from two vertebrate livers (beef and chicken) have been purified to apparent homogeneity, many intriguing problems concerning the nature of the purification process remain to be examined. These include the nature of the unknown substances which bind to the affinity columns, the multiple peaks of activity observed on isoelectric focusing and the variation in isoelectric points of the reductases from various animal and bacterial species. Using the highly purified reductases from bovine and chicken liver, investigations are continuing on the structure (amino acid sequence and x-ray crystallography) and function of these enzymes.

Methods Employed: Affinity chromatography, using a chromatographic system based on the drug methotrexate coupled to sepharose, has been used to absorb dihydrofolate reductase from relatively crude liver extracts. However in order to obtain the homogeneous, substrate-free reductase, additional purification procedures involving gel filtration and isoelectric focusing are required. Structural studies on the enzyme involved the use of automatic amino acid analysis using the general procedures of Moore and Stein. Sequence analyses involving Edman degradation were performed with an automated sequenator. The phenylthiohydantoin-amino acid derivatives sequentially liberated were identified by gas chromatography after trimethylsilylation. Additional methods of chemical and enzymatic analysis involved the use of spectrophotometry and fluorescence spectroscopy.

Major Findings: Although no physiological modifiers have yet been found, certain modifications of the singly sulfhydryl group, which our sequence studies have shown to be located at position 11 of the amino terminal end of the single polypeptide chain, are capable of increasing the enzymatic activity by a factor of 5 to 10-fold. This activation which can be achieved by reactions with a variety of reagents, e.g., stoichiometric amounts of organic mercurials and iodine as well as by treatment with the chaotropic agents, urea and thiourea and now reaction with tetrathionate. In this process one mole of thiosulfate becomes covalently attached to the single sulfhydryl group of the enzyme according to the following equation in which the inner S is shown labeled:



(1)

The identity of the attached thiosulfate was established by obtaining 1:1 binding independently with both inner and outer labeled [^{35}S] tetrathionate. This established that it was indeed $S_2O_3^{2-}$ which binds and not SO_3^{2-} as is the case when tetrathionate reacts with free cysteine.

The thiosulfate form of the enzyme has 5- to 10-fold more activity as measured with its normal substrate and cofactor, dihydrofolate and TPNH. With folate and TPNH a slight increase (approximately 2-fold) is seen while with dihydrofolate and DPNH, a 20% decline in activity occurs. Modification also changes the K_m's with increases for TPNH and dihydrofolate and decreases for folate and DPNH, when measured near their respective pH optima. Although the thiosulfate enzyme is stable for a week at 4° or at -80° for at least several months, it is far more sensitive to thermal inactivation than the untreated enzyme. Both dihydrofolate and TPNH afford some protection, dihydrofolate being by far the most effective decreasing K_1 by a factor of 15 to 20-fold.

The activation of chicken liver dihydrofolate reductase by tetrathionate is, thus far, unique since every known case of enzyme modification by this reagent results in inhibition. In the case of the beef liver enzyme, tetrathionate apparently does not react with the reductase; neither activation or inhibition is observed. These differences in reactivities toward tetrathionate as well as with the organic mercurials would appear to reside in the location and microenvironment surrounding their respective cysteine residues.

The amino acid sequence of the chicken liver dihydrofolate reductase is homologous to that of the beef liver enzyme as well as to that of the L1210 mouse lymphoma and sarcoma-180. This is particularly true of the first 25 residues from the amino terminal end where the cysteine residue is located. However, only the chicken liver enzyme has a cysteine at position 11 whereas all the other animal reductases sequenced to date show a cysteine at position 6. Similarly, the other animal enzymes show a proline at position 3 but the chicken enzyme has an aspartate at this position. Cysteine-6 in the bovine reductase must be less accessible or chemically less reactive than the corresponding residue at position 11 in the chicken liver enzyme. Not only is this evident from the tetrathionate studies, modification of the bovine enzyme by organic mercurials occurs with some difficulty and, once modified, the bovine enzyme exhibits less activation and becomes quite unstable. It is not known, at present, if similar correlations between enzymatic properties and certain aspects of the amino acid sequences, as is evident from our studies on the bovine versus chick reductase, can be extended to the other animal dihydrofolate reductases. However, by contrast, bacterial reductases which contain one or more cysteine residues are inhibited by organic mercurials. The S faecium reductase contains a single cysteine residue which is not located in the N-terminal 25 amino acid residues. Nearly stoichiometric amounts of pHMB lead to complete inactivation of this enzyme. E. coli dihydrofolate reductase is inactivated by certain thiol reagents. The two reactive cysteine residues are located at positions 85 and 152. Thus it is tentatively concluded that, for animal dihydrofolate reductases stable to such treatment, enzyme activation by organic mercurials is directly related to the presence of a cysteine residue in the amino-terminal region of their amino acid sequence.

Significance to Biomedical Research and the Program of the Institute: Dihydrofolate reductase is not only a key enzyme in the metabolism of folic acid, it is also of major importance in the synthesis of DNA via the biosynthesis of thymidylate. Thus an inhibition of dihydrofolate reductase causes a depletion of tetrahydrofolate leading in turn to a deficiency in thymidylate. Such a deficiency can lead to a cessation of growth in rapidly proliferating cells such as tumor cells. Since a variety of dihydrofolate reductase inhibitors are clinically useful as anti-bacterials, anti-protozoals, immunosuppressants and anti-neoplastic agents, an understanding of the structure and function of this enzyme would be of basic importance in both normal metabolism and clinical medicine. As yet, many of these basic questions remain to be answered.

Proposed Course of Project: Now that the basic physical properties of the homogenous proteins are known for both the beef and chicken dihydrofolate reductases, studies are continuing towards the elucidation of the structure of the enzymes in terms of the amino acid sequences and three dimensional structure via x-ray analysis. In addition, studies will continue to be directed toward the investigation of the interaction of drugs and cofactors with the purified reductases.

Publications:

Subramanian, S. and Kaufman, B.T.: Interaction of methotrexate, folates and pyridine nucleotides with dihydrofolate reductase: Calorimetric and spectroscopic binding studies. Proc. Natl. Acad. Sci. (USA) 75: 3201, 1978.

Barbehenn, E.K. and Kaufman, B.T.: Activation of chicken liver dihydrofolate reductase by tetrathionate. Biochem. Biophys. Res. Commun. 85: 402, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM 15300-14 LNE

PERIOD COVERED
October 1, 1978, to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biochemical Studies Related to Nutrition

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. G. Bieri, Chief, Nutritional Biochemistry Sec., LNE, NIAMDD

Other: M. C. McKenna, Staff Fellow, Nutritional Biochemistry Sec., LNE, NIAMDD
T. J. Tolliver, Chemist, Nutritional Biochemistry Sec., LNE, NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Nutritional Biochemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	2	3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of the fluorescent pigment lipofuscin, which accumulates in vitamin E deficient rat uterus, showed that the formation of the pigment in this tissue is unaffected by the animal's vitamin A status. It was concluded that vitamin A cannot be a significant component of lipofuscin. Quantification of the pigment by determining the fluorescence of lipid extracts from affected uteri was found not to give a reliable estimate of the extent of lipofuscin deposition. A rapid micro method, utilizing high pressure liquid chromatography, was developed for the simultaneous determination of vitamins A and E in blood plasma and red cells.

PHS-6040
(Rev. 10-76)

Objectives: To determine the nutritional, biochemical and physiological role of essential nutrients for experimental animals. To define the metabolic function of certain nutrients and to study nutritional effects on the tissue levels of various metabolites.

Methods Employed: Rats, hamsters and guinea pigs are fed specially prepared, highly purified diets that contain adequate amounts of each nutrient known to be required by the particular species. The effects of specific deficiencies and imbalances are assessed by measurement of physiological, chemical and enzymological changes in the animal, its tissues and excreta.

Major Findings: Studies were conducted of the fluorescent pigment, lipofuscin, which accumulates in tissues of vitamin E deficient animals. The primary aim was to determine if retinol (vitamin A) contributed to the formation of this pigment, as had been reported by others, and a secondary aim was to evaluate methods for quantifying this pigment. Weanling female rats were fed a basal vitamin E free diet with (a) no retinol (but with retinoic acid which does not accumulate in tissues) (b) a very low retinol content sufficient to promote normal growth but not permit tissue storage of the vitamin (c) a high retinol content. After 3, 5, and 8 months the animals were sacrificed and the uteri removed for analysis. By 3 months, no obvious changes had occurred but by 5 months uteri from vitamin E deficient rats were dark brown with lipofuscin pigment. There was no difference in pigmentation, or in fluorescence under ultraviolet irradiation, whether the animals received low, high or no retinol (i.e., retinoic acid). Chloroform-methanol extracts of the uteri were examined for fluorescence before and after chromatography on Sephadex LH-20. A single peak from the column, described by others as a component of lipofuscin, was isolated and its fluorescence determined. This peak area was 2 to 3 times greater in extracts from vitamin E deficient rats as compared to control rats. Similarly, the total lipid extracts and also the water washings from the lipid extracts had 2-3 times more fluorescence when the animals were vitamin E deficient. In contrast to the great difference (probably more than 100 fold) in lipofuscin pigment seen visually or estimated by fluorescence in deficient and control uteri, we conclude that extracts give a relatively insensitive estimation of lipofuscin in tissues. Also, in vitamin E deficient uteri there was no difference in the amount of lipofuscin found as estimated by visual observation, fluorescence, or by extraction whether rats were fed no retinol, low retinol, or high retinol. It is concluded that retinol is not a significant component of lipofuscin.

In a second area of investigation, we have successfully developed a rapid, micro procedure for simultaneously determining retinol (vitamin A) and α-tocopherol (vitamin E) in blood plasma and red cells utilizing high pressure liquid chromatography. The method reduces the time required for these vitamins from about two days for six samples in duplicate to about two hours. It should have wide application for nutritional assessment and clinical studies, particularly in children. The procedure is currently being applied in studies of the vitamin status of patients with cystic fibrosis, a-betalipoproteinemia, glucose-6-phosphate dehydrogenase deficiency, and thalassemia.

Publications:

(1) Bieri, J.G., Tolliver, T.J., and Catignani, G.L.: Simultaneous determination of α-tocopherol and retinol in plasma and red cells by high pressure liquid chromatography. Am. J. Clin. Nutr., 1979 (in press).

(2) Boyd, M.R., Catignani, G.L., Sasame, H.A., Mitchell, J.R., and Stiko, A.W.: Acute pulmonary injury in rats by nitrofurantoin and modification by vitamin E, dietary fat and oxygen. Am. Rev. Respir. Dis., 1979 (in press).

(3) Carter-Dawson, L., Kuwabara, T., O'Brien, P.J., and Bieri, J.G.: Structural and biochemical changes in vitamin A deficient rat retinas. Invest. Opthalmol. Visual Sci. 18: 437-446, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z 01 AM 15302-09 LNE

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biochemical Studies of Hepatic and Intestinal Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. H. G. Windmueller	Research Chemist	LNE	NIAMDD
OTHER:	Dr. A. Wu	Staff Fellow	LNE	NIAMDD
	Mr. A. E. Spaeth	Chemist	LNE	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Nutrition and Endocrinology

SECTION

Nutritional Biochemistry Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In the rat, liver and intestine were found to be the only sources of the constituent apoproteins of plasma lipoproteins. Intestine produced 19% of the total apolipoprotein pool -- 50-60% of apolipoprotein A-I (apoA-I) and apoA-IV, 16% of apoB, <10% of apoC and <1% of apoE. The quantitative order of synthesis in the intact animal was apoE > apoB > apoA-I > apoC > apoA-IV. ApoA-I and apoA-IV are major constituents of plasma high density lipoproteins, and so intestine contributes importantly to this lipoprotein class. In other studies with in situ autoperfused jejunal segments from fed rats, plasma glutamine was identified as the major respiratory fuel after the lumen was emptied. Glutamine nitrogen was released into portal blood as ammonia and alanine, substrates for urea synthesis in liver, and as citrulline, a substrate for arginine synthesis in kidney. During absorption of a typical meal, intestinal metabolism of luminal glutamine, glutamate and aspartate accounted for 40% of the respiratory CO_2 produced. These studies reveal the importance of glutamate and aspartate in intestinal energy metabolism and the quantitatively significant role of small intestine in processing circulating glutamine, a major nitrogenous end-product from muscle and other tissues.

Objectives: To increase our understanding of the biochemical transformations occurring in the liver and small intestine. To define the nutritional requirements of these organs, to study the effects of hormones and nutritional variables, and to study the physiological relationship of these organs to other tissues in the body.

Methods Employed: Surgical and biochemical techniques are used. Surgically prepared animal preparations include isolated, perfused rat livers; isolated vascularly perfused rat small intestine; isolated segments of rat small intestine autoperfused in situ; supra-diaphragmatic rat preparations; and intact animals with various in-dwelling catheters -- in the duodenum, mesenteric lymph duct, bile duct, and jugular vein. The metabolic and transport activity of the organs, isolated or in situ, are studied by a combination of biochemical and radiochemical techniques. The fate of labeled metabolites is quantitatively determined and the concentration of various blood and tissue metabolites is measured enzymatically. Various blood and lymph proteins are isolated by a combination of ultracentrifugal and electrophoretic methods, and their rates of biosynthesis determined from the rates of incorporation of appropriate radioactively-labeled precursors. Also, tissue enzymes are localized, isolated and characterized.

Major Findings: (A) Role of individual organs in apolipoprotein synthesis. We have made a quantitative assessment of the role of liver and intestine in the production of the protein moieties of plasma lipoproteins. Circulating lipoproteins are high-molecular weight complexes that serve as transport vehicles for various lipids (sterols, triacylglycerols, phospholipids, fat-soluble vitamins) among the body tissues. Elevated concentrations of certain plasma lipoproteins and depressed levels of others are associated with abnormal deposition of lipids in arterial endothelial cells (arteriosclerosis). We still know relatively few details regarding lipoprotein structure, synthesis, and metabolism, owing largely to the complex nature of these vary large and heterogeneous particles. Rat plasma lipoproteins contain at least 10 different polypeptides or apolipoproteins, largely analogous to the constituent apolipoproteins of human plasma. Physiological functions have been deduced for some of these proteins -- in the assembly and transcellular transport of these complexes and in their metabolism in blood and other tissues. The functions of some of these proteins remain obscure.

In previous work from this and other laboratories, liver and intestine were identified as sources of the plasma apolipoproteins, and the pattern of synthesis for each of these organs was established. Liver was shown to be capable of synthesizing all of the known apoproteins. Most, but not all, could also be synthesized by small intestine. The relative amount of the various proteins made in each tissue was very different. We have now shown that liver and intestine are the only rat tissues which produce plasma apolipoproteins. This was shown by studies in rats in which the small intestine was resected and the liver surgically removed from the circulation. In such animals, no [^{3}H]leucine, a radioactive protein precursor, was incorporated into any of the apolipoproteins. We next developed two methods to

determine the relative amounts of each of the apoproteins synthesized by each organ.

In Method I, plasma apolipoproteins of intestinal and hepatic origin were differentially labeled in vivo by simultaneously administering [^{3}H]leucine intraduodenally and [^{14}C]leucine intraportally. Thus, intestinally made proteins were preferentially labeled with ^{3}H and hepatically made proteins with ^{14}C. The ^{3}H/^{14}C ratio of each plasma apoprotein was determined. For reference purposes, the ratio found in plasma albumin characterized a protein made in liver and the ratio found in selected apolipoproteins isolated from mesenteric lymph characterized a protein made by the small intestine. A mathematical treatment of the results was developed from which the relative organ contributions could be estimated. In Method II, the patterns of apolipoprotein synthesis, defined as the relative amounts of labeled leucine incorporated into the individual proteins, were determined in isolated, perfused livers, in an isolated, auto-perfused jejunal segment preparation in situ, and in intact rats, from label administered into the jugular vein. By correlating the observed values by multiple regressive analysis, the relative organ contributions needed to account for the pattern observed in the intact animal could then be calculated. Results from the two complementary methods, both applied in fat-fed animals, were in good agreement.

The quantitative order of apolipoprotein synthesis in intact rats was apolipoprotein E (apoE) > apoB > apoA-I > apoC > apoA-IV. Intestine synthesized 19% of the entire apolipoprotein pool and liver 81%. The percent intestinal contribution to individual proteins was as follows: apoA-IV, 59; apoA-I, 56; apoB, 16; apoC-II, 10; apoC-III-0, 7; apoC-III-2(-1), <10; apo-C-III-3, <1; and apoE, <1. Little or no apoC-I was derived from intestine. Since apoA-I and apoA-IV are major protein constituents of plasma high density lipoproteins(HDL), these results show that the small intestine contributes importantly to this lipoprotein class, the plasma concentration of which correlates with resistance to arteriosclerosis.

(B) Relationship of lipoprotein structure to catabolism by liver: In a collaborative project with Dr. R. W. Mahley, we measured the rates of catabolism by isolated perfused rat livers of various human and rat lipoproteins before and after selective modification of lysine residues by acetoacetylation. Acetoacetylation of human low density lipoprotein greatly enhanced the rate of clearance by the livers. It was demonstrated that Kupffer cells were largely responsible for the uptake. Similar results were obtained with human HDL_3. In contrast, acetoacetylation of two apoE containing lipoproteins retarded their clearance from blood. These results indicate that the hepatic recognition and removal process is specific for a property acquired by only certain lipoproteins after acetoacetylation. Further work is needed to relate these findings to the normal clearance of plasma lipoproteins in vivo.

(C) Metabolism of vascular and luminal substrates by intestine: In recent work from this laboratory, we identified circulating glutamine and ketone bodies as the major respiratory fuels in vivo for small intestine of rats in the post-absorptive state. Glucose made only a small energy contribution, in contrast to previous dogma. These studies were made possible by use of an autoperfused jejunal segment preparation in situ with totally collected venous drainage, a preparation which permits control of luminal contents, arteriovenous difference and blood flow rate measurements, and the performance of quantitative metabolic studies with labeled substrates administered either vascularly or luminally. These studies have now been extended to animals in the fed state. With food flushed from the lumen, O_2 uptake and CO_2 release by intestine were 50% higher than in the post-absorptive state. Arterial glutamine remained the single most important energy source, accounting for nearly 50% of the respired CO_2. Glucose, which is converted largely to lactate and alanine, contributed only 10%. The remaining CO_2 was derived from ketone bodies, lactate, and apparently some from endogenous substrates. The glutamine nitrogen was released back into blood as ammonia (38%), citrulline (29%), alanine (24%), proline (7%) and glutamate (2%). Utilization of arterial glutamine was about 4-times as great for the whole small intestine as in the post-absorptive state, and equalled about 120 μmol/h. This is equivalent to the total blood glutamine content every 10 min. and represents a major pathway for metabolism of blood glutamine, an important end-product of nitrogen metabolism in skeletal muscle and brain. The ammonia and alanine proceed to the liver where the nitrogen is converted to urea. The citrulline can serve as a substrate for arginine synthesis by the kidney.

O_2 consumption by jejunal segments increased only slightly when the lumen was perfused with a simulated meal of glucose and amino acids, showing that only a small fraction of the energy expended by intestine is needed to support the transport of luminal substrates into blood.

The contribution of luminal substrates to intestinal energy production was also determined. Glucose was transported at a high rate from lumen to blood, but only 3% of it was metabolized by the transporting cells with a CO_2 yield that accounted for only 6% of the total produced. On the other hand, aspartate, glutamate, and glutamine in the perfused meal accounted for 39% of the total CO_2 produced. These results emphasize the importance of certain amino acids as energy fuels in the intestine and the sparing effect of luminal substrates on the utilization of arterial substrates following meal eating. The distribution of glucose carbon among its various metabolic products was found to be similar whether glucose was administered vascularly or luminally, in contrast to recent statements in the literature based on work with less physiological intestinal preparations in vitro.

These and our earlier studies have focused attention on the significant role of the small intestine in nitrogen metabolism. High blood levels of aspartate and glutamate are known to be toxic. By metabolizing dietary aspartate, glutamate, and glutamine to ammonia, CO_2, alanine, citrulline,

and lactate, the intestine protects the animal and converts the nitrogen into compounds readily used by the liver and kidneys. The extensive metabolism of circulating glutamine not only provides much of the energy for this organ but serves to process the nitrogen released from peripheral tissues as non-toxic glutamine into alanine and into ammonia, which is efficiently removed from the portal circulation by the liver and converted into urea, thereby preventing exposure of other tissues to this toxic compound.

Significance of Biomedical Research and the Program of the Institute: These studies add to our understanding of hepatic and intestinal function; in particular, they help to define the roles of these organs in the biogenesis of circulating lipoproteins, in the metabolism of circulating and luminal substrates, and in the complex metabolic interrelationships that exist among the various body organs. In addition, they provide the methods and basic information for subsequent analyses of hepatic and intestinal function under altered and pathological conditions.

Proposed Course: To determine if the roles of intestine and liver in lipoprotein biogenesis are altered under different dietary and physiological circumstances, e.g., fasting, high cholesterol intake, high intake of saturated fat; to further exploit the auto-perfused jejunal segment preparation in answering questions related to the transport, secretory and metabolic activity of the small intestine.

Publications:

(1) Wu, A.-L. and Windmueller, H.G.: Relative contributions by liver and intestine to individual plasma apolipoproteins in the rat. J. Biol. Chem. 254, 1979 (in press).

(2) Mahley, R.W., Weisgraber, K.H., Innerarity, T.L. and Windmueller, H.G.: Accelerated clearance of low-density lipoproteins and retarded clearance of E apoprotein-containing lipoproteins from plasma of rats after modification of lysine residues. Proc. Natl. Acad. Sci., U.S.A. 76: 1746-1750, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-15400-05-LNE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Hormones, Lipoprotein Lipase and Lipid Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Scow	Chief, Section on Endocrinology	LNE	NIAMDD
Other:	Peter M. Spooner	Staff Fellow	LNE	NIAMDD
	Sidney S. Chernick	Scientist Director	LNE	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Endocrinology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.8	1.8	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin regulation of lipoprotein lipase (LPL) in fat cells was studied in fully differentiated 3T3-L1 adipocytes. Insulin ($<10^{-7}$ M) elicited a rapid increase (10x) in release of LPL from cells to medium, and a slower increase (5x) in total LPL in the cells. The insulin-induced increase in LPL activity in cells required glucose. Glucose added to cells incubated 20 hr with insulin in hexose-free medium elicited a full response to insulin in 2 hr. The latter was prevented by cycloheximide but not actinomycin D, whereas the response in cells incubated with glucose for 24 hr was blocked completely by either inhibitor. It is concluded insulin regulates LPL through three major actions: 1) It stimulates release of LPL from fat cells. 2) It stimulates gene expression and RNA synthesis independently of effects on hexose and amino acid transport. 3) It stimulates hexose transport which enhances expression of nuclear effects of insulin at the translational level.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives

To determine the influence of hormones, lipoprotein lipase and other factors on the metabolism of lipid in liver, adipose tissue, heart, mammary gland, crop sac and other tissues. To determine how hormones regulate lipoprotein lipase activity in tissues. To develop a culture system for mammary and adipose cells that support differentiation of functions in response to hormones in vitro.

Methods Employed

Animals are deprived of one or several endocrine glands and treated with hormones or other substances. Effects of the above on lipid metabolism and lipoprotein lipase activity in different tissues are determined with in vivo and in vitro studies using conventional and radioisotopic techniques. Effects of hormones added in vitro are also studied. Cells isolated from mammary, adipose and other tissues are grown in culture and used for study of mechanism of action of hormones.

Major Findings

Insulin Regulation of Lipoprotein Lipase Activity and Release in Fat Cells

In the previous year we established that the 3T3-L1 mouse fibroblast cell line, which differentiates into fat cells in culture, provides an excellent model system for examining hormonal regulation of lipoprotein lipase (LPL). We found that 1) LPL activity was extremely low in undifferentiated fibroblasts and increased ∿100-fold in fully differentiated fat cells; 2) biochemical properties of LPL in these cells were identical to that found in adipose tissue; 3) appearance of LPL was unaffected by the concentration of substrate, lipoprotein triacylglycerol, in the culture medium during differentiation, and 4) LPL activity in these cells could facilitate triacylglycerol deposition, independent of de novo fatty acid synthesis, during development of the preadipocyte. Since the appearance of LPL served as one of the earliest and most reliable markers of the differentiated state, we were able to describe the effects of a number of hormonal agents (insulin, prolactin, prostaglandin $F_{2\alpha}$, methylxanthine) on the development process. Insulin proved to be the most potent and important factor regulating development of LPL activity and deposition of triacylglycerol in these cells.

During the past year we have studied the mechanisms by which insulin regulates LPL in mature, fully differentiated 3T3-L1 fat cells. Low, physiologically relevant concentrations of insulin (10^{-10} to 10^{-7} M) exerted two effects on LPL in these cells, an increase in rate of release of the enzyme from cells to the medium (∿10 fold in 15 min), and a slower increase in total enzyme activity in the cells (3- to 10-fold in 2 days). Prolonged treatment with insulin also resulted in additional increases in enzyme

release and a 10-fold increase in the fractional rate of release of enzyme per hour. Both effects mimic previously described actions of insulin on LPL activity in adipose tissue in vivo.

Stimulation of cellular lipoprotein lipase activity by insulin was dependent on the presence of glucose in the incubation medium. Although half-maximal increases in total activity required 12 hours of insulin treatment in glucose-containing medium, removal of either glucose or insulin resulted in loss within 90 min of >50% of the stimulation in enzyme activity. Addition of glucose to cells incubated 20 hours with insulin in carbohydrate-free medium elicited a rapid and full response to insulin in 2 hours. Fructose, mannose and glucosamine could substitute for glucose in supporting this rapid response in insulin-pretreated cells, but only glucose, mannose and fructose would support the effect in 24 hour-long incubations with insulin. Induction of increase in LPL by glucose in insulin-pretreated cells was prevented by cycloheximide, but not by actinomycin D. In contrast, inhibitors of either protein or RNA synthesis completely blocked the response to insulin in cells incubated 24 hours with glucose.

On the basis of these findings, we propose that insulin regulates lipoprotein lipase in fat cells through three major actions: 1) Insulin elicits rapid release of enzyme from cells through mechanisms which are independent of energy metabolism, protein synthesis, and functioning cytoskeletal elements. 2) Insulin stimulates gene expression and mRNA synthesis independently of any effect on hexose or amino acid transport. 3) Insulin stimulates expression of nuclear effects of the hormone at the translational level by increasing hexose uptake and levels of regulatory glycolytic metabolites within the cells. Induction of the enzyme and its release thus appear part of a generalized pleotrophic growth response mediated by separable effects on membrane function and macromolecular synthesis.

Significance to Biomedical Research and the Program of the Institute

Lipoprotein lipase is necessary for the uptake of triacylglycerol from blood by extrahepatic tissues. Earlier studies in this laboratory showed that the enzyme acts in capillary endothelium to hydrolyze triacylglycerol to partial glycerides and fatty acids which cross the capillary to be utilized by tissue cells. The present studies contribute information concerning the regulation and synthesis of lipoprotein lipase in adipocytes, the probable source of this enzyme in adipose tissue. It is evident that this enzyme plays a major role in the deposition of triacylglycerol in adipose tissue (see Project No. Z01-AM-15401-07-LNE).

Proposed Course

Studies are currently in progress to 1) identify hexose metabolites that regulate enzyme synthesis at the translational level and determine their mode of action, 2) determine the mechanism by which catecholamines and fatty acids inhibit LPL activity in these cells, and 3) produce specific antibodies to LPL isolated from 3T3-L1-cells.

Publications:

Spooner, P. M., Chernick, S. S., Garrison, M. M., and Scow, R. O.: Development of lipoprotein lipase activity and accumulation of triacylglycerol in differentiating 3T3-L1 adipocytes. J. Biol. Chem. 254: 1305-1311, 1979.

Van Obberghen, E., Spooner, P. M., Kahn, C. R., Chernick, S. S., Garrison, M. M., Karlsson, F. A., and Grunfeld, C.: Antibodies against the insulin receptor mimic a long-term insulin effect: Increase in lipoprotein lipase activity in 3T3-L1 fatty fibroblasts. Nature (In press) 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-15401-07-LNE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (30 characters or less)

Transport of lipids, hormones and enzymes in tissues, cells and membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Scow	Chief, Section on Endocrinology	LNE	NIAMDD
Other:	E. Joan Blanchette-Mackie	Research Biologist	LNE	NIAMDD
	Mary G. Wetzel	Staff Fellow	LNE	NIAMDD

COOPERATING UNITS (if any)

Dr. Louis C. Smith, Division of Atherosclerosis and Lipoprotein Branch, Department of Medicine, Baylor College of Medicine, Houston, Texas 77030

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Endocrinology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	2.5	2.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our group has developed a model for transport of lipid from blood to tissue by lateral movement in a continuous interface composed of cell membranes. Lipid monolayer studies showed that lipids formed by action of lipase on triacylglycerol located and moved in the interface. Recent morphological studies of adipose tissue of suckling rats showed continuity between the outer leaflet of plasma membrane of endothelial cells and that of adipocyes, and continuity between plasma and intracellular membranes in both endothelial and fat cells. Aggregations of lipolytic products, appearing as lamellar whorls within the continuum, were formed from chylomicrons in capillaries of tissue from fed rats and from lipid droplets in adipocytes of tissue from fasted rats. Changes in distribution of lamellae associated with incubation of fixed tissues demonstrated movement of lipolytic products from their respective sites of formation. Thus, the lamellae mark in fixed tissue the membrane continuum proposed for transport of lipid from blood.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives

To study structure and metabolism of cells that synthesize and secrete lipids, hormones and enzymes. To study transport of lipids, hormones and enzymes across capillary endothelium, basement membrane and extracellular space in different tissues. To study uptake of lipids, hormones and enzymes by different cells. To study structure of cellular membranes and their role in lipid metabolism. To correlate structural with biochemical changes induced experimentally in cells and tissues. To study effects of lipolytic enzymes on lipid monolayers and lateral movement of products in the interface.

Methods Employed

Animals of different species and ages and in various physiological states (e.g., fasting, pregnant) are used to study with biochemical and morphological techniques synthesis, and storage or secretion of triacylglycerol in adipose tissue, heart, mammary gland, liver and intestines, and uptake and utilization of blood triacylglycerol by adipose tissue, heart, mammary gland and muscle. Morphology of cellular membranes and other structures are studied with the electron microscope and the freeze-fracture technique.

Monolayer techniques are used to study action of lipoprotein and other lipases on triacylglycerol and movement of lipolytic products in the gas-water interface.

Major findings

Lipid Transport between Blood and Tissue by Lateral Movement in Cell Membranes

We proposed several years ago a model for transport of lipid from chylomicrons to tissue cells by lateral movement in cell membranes. The model requires 1) conversion of chylomicron triacylglycerol to amphipathic lipids, fatty acids and monoacylglycerol by lipoprotein lipase at the capillary surface, 2) location and lateral movement of these products in a continuous interface consisting of the chylomicron surface film and the external leaflets of plasma and intracellular membranes of endothelial and parenchymal cells, and 3) removal of these products from the interface in endoplasmic reticulum of cells where they are reesterified to triacylglycerol and separate from the interface, forming lipid droplets between leaflets of endoplasmic reticulum.

Morphological studies reported earlier of chylomicrons incubated with lipoprotein lipase and limited albumin showed that lipolytic products accumulated in the interface between triacylglycerol and the aqueous medium, and extended the chylomicron surface film as a monolayer lining and

spiraling within aqueous spaces that developed in the chylomicron core. Biochemical studies reported last year on effects of lipoprotein lipase on triacylglycerol at an argon-water interface, using a tricomparted trough, showed that lipolytic products located and spread immediately throughout the interface. As they crowded the interface, surface pressure increased, lipolysis slowed down, and substances with lower spreading pressures were displaced from the interface. Addition of albumin to the aqueous subphase, to serve as a trapping agent, increased markedly desorption of fatty acids and monoacylglycerol from the interface, and thereby lowered surface pressure and enhanced lipolysis. Lateral movement of these lipids in the interface was demonstrated when lipoprotein lipase and albumin were added to separate compartments.

The concept that there is an interfacial continuum between chylomicrons and tissue cells during uptake of triacylglycerol from chylomicrons is supported by observations in rat tissues incubated after being fixed with glutaraldehyde. Biochemical studies showed that lipoprotein lipase is active in fixed tissue. Morphological studies of glutaraldehyde-fixed adipose tissue incubated at 38°C showed lamellar structures in vacuoles and vesicles of capillary endothelium and in spaces between endothelium and pericytes. These structures were not found in unincubated tissue. At the time of the study we concluded that the lamellar structures represented lipolytic products at sites of hydrolysis, and that hydrolysis occurred within vacuoles and vesicles of endothelial cells and in subendothelial spaces. In view of our recent finding that fatty acids and monoacylglycerol can transfer from the site of hydrolysis by lateral movement in the interfacial plane between triacylglycerol and water, we now conclude that the lamellar structures represent primarily products, not sites, of hydrolysis. Since the main source of triacylglycerol for lipoprotein lipase activity in fixed tissue is chylomicrons attached to capillary endothelium, it seemed likely that in the above studies hydrolysis occurred at the surface of chylomicrons and lipolytic products moved across the capillary endothelium within the luminal leaflet of membranes of vesicles and vacuoles, and when their movement was impaired they formed lamellar structures within vesicles and vacuoles.

Lamellar structures were found also in mammary gland of lactating rats injected with chylomicrons, in capillary endothelium, in spaces between cells, and associated with cellular projections. When fixed mammary tissue was incubated at 38°C, lamellar structures were found near mammary secretory cells, indicating movement of lipolytic products during incubation. The continuity of plasma membrane with layers of lamellar structures suggested the lamellae might be formed as lateral extensions of membranes.

Heart derives much of its energy from fatty acids taken up from chylomicrons. Recent morphological studies of heart from adult rats injected with chylomicrons showed the presence of lamellar structures in capillary endothelium, in the spaces between endothelium and other cells, at the surface of myocytes, and associated with the tubular system and mitochondria of myocytes. Incubation of heart tissue after being fixed with glutaraldehyde increased the amount of lamellae seen at all sites. The

lamellae associated with mitochondria could represent lipolytic products derived from fat stores in myocytes. This possibility is under study.

Adipocytes undergo rapid development and growth in epididymal and parametrial adipose tissue of young suckling rats. They readily take up fatty acids from chylomicrons during feeding, and release fatty acids from intracellular fat stores to the bloodstream during fasting. Adipose tissue of 6-11 day old suckling rats was processed for electron microscopy with special fixative procedures developed to retain lipid in tissue. Morphological study of these tissues showed cytoplasmic processes of endothelial cells that extended to adipocytes and similar processes of adipocytes that extended to other cells, continuity between the outer leaflet of plasma membrane of endothelial cells and that of fat cells, and continuity between plasma and intracellular membranes in both endothelial and fat cells. These findings demonstrate the presence of a structural continuum between and within cells.

Many spiraling lamellae were found in sections of adipose tissue of suckling rats. The lamellae were continuous with membranes of both capillary endothelium and adipocytes. Experiments were designed to determine the relationship between distribution of lamellae and lipolytic activity in capillaries and fat cells. Epididymal and parametrial fat pads were taken from fed and fasted rats, and also from fed rats injected intravenously with chylomicrons. Lamellae were found primarily in and near capillary endothelium in adipose tissue from fed animals, and they increased in size and number and also appeared near fat cells when the tissue was incubated at 24°C after being fixed with glutaraldehyde. The lamellae were considerably larger and more numerous in tissue from fed rats injected with chylomicrons. In tissue from fasted animals, lamellae were found primarily in and near fat cells, and they increased in size and number and appeared near capillaries when the tissue was incubated after being fixed. Lamellae were absent in adipose tissue of fed rats when the tissue was incubated with 5 mM glucose and 10^{-7} M insulin before being fixed. Biochemical studies of adipose tissue from suckling rats injected with chylomicrons containing triacyglycerol labeled with [^{3}H]oleic acid showed that chylomicron triacyglycerol was hydrolyzed to fatty acids when unfixed tissue was incubated in Tyrode's solution (pH 7.4) at 38°C, and that all of the fatty acids produced were immediately reesterified to triacylglycerol when glucose and insulin were added to the incubation medium. The absence of both lamellae and fatty acids in tissues incubated with glucose and insulin indicate that the lamellae are composed of lipolytic products.

We conclude that the lamellae in developing adipose tissue represent aggregations of lipolytic products within the interfacial continuum of membranes, and that they were formed during tissue preparation from chylomicrons in tissue from fed rats, and from fat stores in adipocytes of tissue from fasted rats. The changes in distribution of lamellae associated with incubation of fixed tissue, with lamellae first appearing near adipocytes in tissue from fed rats and near capillaries in tissue from fasted rats, demonstrate movement of lipolytic products from their respective sites

of formation. Thus, the lamellae mark in fixed tissue the membrane continuum proposed for transport of lipid from blood.

Significance to Biomedical Research and the Program of the Institute

Uptake of triacyglycerol from chylomicrons and very low density lipoproteins (VLDL) requires hydrolysis of triacylglycerol to fatty acid and monacylglycerol by lipoprotein lipase acting at the luminal surface of the capillary endothelium. Some of the fatty acids formed are released to the bloodstream while the rest transfer with monoacylglycerol to the tissue. Our studies on the clearance of chylomicrons from blood have led to the development of a model for transport of lipid from blood to tissue by lateral movement in a continuous interface composed of cell membranes. Our earlier studies demonstrated that products of lipolysis immediately locate and move within the interface. Our recent findings, reported above, provide structural evidence for an interfacial continuum of membranes extending from the luminal surface of capillaries to the endoplasmic reticulum of parenchymal cells, where lipolytic products are reesterified to triacylglycerol and separate from the interface. A similar route is probably used for transport of other amphipathic substances (e.g., cholesterol, phospholipids, certain amino acids, steroid hormones) from blood to tissue cells and transport of fatty acids within cells from lipid stores to mitochondria. Abnormal deposition of lipids in blood vessels and other tissues may also involve lipid transport by lateral movement in cell membranes.

Proposed Course

The action of lipoprotein lipase on chylomicrons in capillaries and transport of lipids between and within cells will be studied in adipose tissue, heart, mammary gland and brown fat with biochemical and morphological techniques, including freeze-fracture and radioautography. The role of lipoprotein lipase in the formation of high density lipoproteins (HDL) from chylomicrons will be studied in vitro. The lipid monolayer technique will be used to study action of various lipases on triacyglycerol and phospholipids, and movement of different lipids in the interfacial plane.

Publications:

Scow, R. O., Blanchette-Mackie, E. J., and Smith, L. C.: Transport of lipid across capillary endothelium. Fed. Proc. (In press) 1979.

Scow, R. O., Desnuelle, P., and Verger, R.: Lipolysis and lipid movement in a membrane model: Action of lipoprotein lipase. J. Biol. Chem. (In press) 1979.

Smith, L. C., and Scow, R. O.: Intravascular metabolism of lipoproteins: Chylomicrons. Mechanism of transfer of the lipolytic products to cells. In Eisenberg, S. (ed.) Progress in Biochemical Pharmacology: Lipoprotein Metabolism Basel, Karger AG (In press) 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-15402-05 LNE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Pharyngeal lipase and digestion of dietary fat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dr. Robert O. Scow, Chief, Section on Endocrinology LNE NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Endocrinology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

INACTIVE

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-15403-01-LNE

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (30 characters or less)

Diabetes, insulin and blood vessels

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Scow	Chief, Section on Endocrinology	LNE	NIAMDD
Other:	Sidney S. Chernick	Scientist Director	LNE	NIAMDD

COOPERATING UNITS (if any)

Dr. Alli Reinilä, Department of Pathology, University of Oulu, Finland
Dr. Hans K. Åkerblom, Department of Pediatrics, University of Oulu, Finland

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Section on Endocrinology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.7	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Kinetics of insulin removal from peripheral circulation suggested that the first step in uptake of insulin by tissues might be binding of insulin to specific receptors on the luminal surface of capillary endothelium. To study this possibility, methods were developed for isolating capillaries from adipose tissue and measuring binding of ^{125}I-insulin to capillaries. The relationship between insulin receptors of capillary endothelium and receptors of fat cells is under study. Lipid accumulation in muscular arteries of diabetic rats was studied with electron microscopy. Tissues were taken 4 days after induction of diabetes with streptozotocin. Lipid deposits were found in medial smooth muscle cells, near mitochondria and sarcoplasmic reticulum, in muscular arteries in 9 of 24 diabetic rats and in 0 of 17 normal rats. The amount of lipid deposited was closely related to plasma FFA concentration. Thus, lipid deposition in muscular arteries during acute diabetes may result from high concentrations of FFA in plasma.

HHS-6040
(Rev. 10-76)

Project Description:

Objectives

To study the effect of diabetes, insulin and various other hormones on deposition of lipids in muscular and elastic arteries of experimental animals. To study binding of insulin to capillary and fat cells of adipose tissue. To evaluate the possibility that transport of insulin from blood to fat cells involves 1) formation of insulin-receptors complex at luminal surface of capillaries and 2) transfer of the complex from capillary to fat cell by lateral movement in a continuum of cell membranes (see Project No. Z01-AM-15401-07-LNE), and that insulin receptors transfer from fat cells to capillary endothelium by the same mechanism.

Methods Employed

Diabetes is induced in experimental animals by injection of streptozotocin or by total pancreatectomy. Effect of diabetes and injected hormones on deposition of lipid in arteries is studied with electron microscopy, radioautography, and biochemical analyses. The source and kind of lipid deposited, and the site of deposition in the vessels is also studied with morphological and biochemical techniques.

Segments of capillary and fat cells are isolated from adipose tissue of rats in different physiological states and used to study binding of insulin to capillary endothelium and fat cells.

Major Findings

Insulin Receptors of Isolated Capillaries

The kinetics of insulin removal from peripheral circulation gave rise to the suggestion that the first step in insulin uptake by some tissues might be the binding of hormone to specific receptors on the luminal surface of capillary endothelium. In an effort to quantitate this phenomenon and to identify the physiological factors that affect insulin receptors on capillaries we have successfully prepared isolated capillaries and measured their binding of ^{125}I-insulin.

Capillaries were isolated from rat parametrial fat pads by digestion with collagenase, separation of stromo-vascular cells from fat cells by low speed centrifugation, and collection of capillaries (and other small blood vessels) on membranes or meshes of known pore size. Insulin binding and degradation were determined by procedures developed at NIAMDD. DNA was determined by a microfluorimetric procedure on the same sample used in the binding study.

The results indicate that isolated capillaries bind ^{125}I-insulin in a time-dependent manner. Specific binding reached a steady state at 23°C in 12 hours. The amount of insulin bound was a function of the DNA content of

the sample. The amount of binding per unit DNA was greater in the larger vascular fractions (retained by 50 μm mesh) than in the smaller fractions (passed through 50 μm mesh but retained by 8 μm membrane). The free cell and cell fragment fraction (passed through 8 μm filter and collected by centrifugation at 200 g for 3 min) had the least capacity for binding insulin per unit DNA. Degradation of insulin by isolated capillaries was also time-dependent and about 10% of the added label was TCA-soluble in 1 hour.

Experiments in progress are designed to relate binding of insulin to its biological activity (increases glucose oxidation and lipogenesis) and to explore the relationship between the insulin receptor concentration of fat cells and that of capillaries in adipose tissue. Initial studies suggest that fasting for 3 days may cause a doubling of insulin binding by isolated capillaries of adipose tissue.

Since the techniques for measuring insulin binding by isolated adipocytes and that by capillaries differ greatly, it is not advisable at present to compare the binding capacities of the preparations. Efforts are being made to relate the binding capacities of fat cells and capillaries from the same tissue in a variety of conditions and to develop a means for quantitative comparison.

Accumulation of Lipid in Muscular Arteries of Short-Term Diabetic Rats

Accumulation of lipid in muscular (pulmonary, coronary and tibial) and elastic (aorta and pulmonary) arteries of diabetic rats was studied with the electron microscope. Diabetes was induced with streptozotocin, and tissues were taken 4 days later. Lipid deposits were found in medial smooth muscle cells, near mitochondria and sarcoplasmic reticulum, in muscular arteries in 9 of 24 diabetic rats and in none of 17 normal control rats. Histochemical analyses demonstrated that the lipid in the cells was triacylglycerol. Lipid deposits were not seen in elastic arteries of either diabetic or normal rats. The diabetic rats had high plasma concentrations of glucose (320 mg/dl), free fatty acids (>0.77 mM), triacylglycerol (1,100 mg/dl) and cholesterol (127 mg/dl), and low plasma concentrations of insulin (<8 μU/ml). The primary differences in plasma between the two groups of diabetic rats were that plasma FFA concentrations were higher and insulin concentrations were lower in rats with arterial lipid deposits. The amount of lipid deposited in the arteries was closely related to plasma FFA concentration, which ranged 0.8-1.2 mM in diabetic rats without deposits and 1.1-2.5 mM in those with deposits. The findings suggest that lipid accumulation in smooth muscle cells of muscular arteries during acute diabetes results primarily from high concentrations of FFA in plasma.

Significance to Biomedical Research and the Program of the Institute

A serious complication of diabetes is arteriosclerosis in muscular arteries of the heart and lower limbs. Although it is generally thought that elevated levels of circulating lipids might be involved, the mechanism of development of macroangiopathy in diabetes is not known. The present study

shows excess accumulation of lipid in muscular arteries of diabetic rats with very high concentrations of FFA in plasma. Whether this would lead to greater lipid accumulation with time, or contribute to development of arteriosclerosis in these arteries needs study.

The mechanism of transport of insulin from blood to tissue cells is also unknown. The study of insulin binding to capillaries has been initiated to gain knowledge concerning this important process.

Proposed Course

The effect of prolonged insulin deficiency and insulin treatment on lipid deposition in muscular arteries and the source of the lipid deposited will be studied. The relationship between insulin receptors of capillary endothelium and receptors of fat cells will be studied in animals in various physiological states.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOI AM 15500-19 LNE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Large-Scale Processing of Biological Materials

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Mr. D.L. Rogerson, Jr. Head, Pilot Plant LNE NIAMDD

Other: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Nutrition and Endocrinology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Production of large quantities of microorganisms for the isolation and purification of biologically important macromolecules and cell particulates and to assist NIH investigators in the scale-up and processing of plant and animal tissues for biological substances such as enzymes, vitamins, alkaloids, etc.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To provide facilities and services for the processing and/or production of large amounts of natural materials, such as bacteria, yeasts, molds, protozoa, animal cells, blood, urine and plant and animal tissues, so that they may be studied under standard laboratory conditions.

Methods Employed: To satisfy the large-scale production needs of NIH investigators, methods are designed for the scaling up of small laboratory experiments utilizing large reaction vessels, extractors, stills, concentrators, centrifuges, etc., so that the processing can be carried out effectively, efficiently and safely. Three 10-liter, one 50-liter, one 100-liter, one 300-liter and one 1100-liter fermentors are utilized for growing microorganisms and animal cells under a variety of conditions. Bacerial cells and yeasts are harvested by means of laboratory or large-scale AS-16P and AS-26P Sharples centrifuges. Protozoa are concentrated in low speed centrifuges. Cells are either supplied as a paste or further processed to yield cell-free suspensions by rupture with a Gaulin homogenizer. In some instances, a specific substance is isolated and partially purified by further processing of the culture supernatant or the cell paste using conventional fractionation procedures and the newer chromatographic techniques.

Major Findings: During the past 12 months, a total of 270 requests were processed for investigators: NIAMDD 145; BoB 45; NCI 37; NHLBI 17; NIAID 10; NICHD 5; NINCDS and NIMH 3 each; NIEHS, NIDR and DRS 1 each and others 2. 28,680 liters of cultures of 22 organisms were produced. These organisms include wild type and mutant strains of Arthrobacter polychromogenes, Aspergillus fumigatus, Bacillus amyloliquefaciens, Bacillus licheni-formis, Bacillus pumilus, Bacillus sublilis, Bordetella pertussis, Candida albicaus, Candida utilis, Chlorella pyriformis, Escherichia coli, Euglena glacilis, Haemophilus influenza types b,c,d,f, Neisseria meningitidis, Providencia alcalifaciens, Rodospirillum rubrum, Saccharomyces cerevisiae, Serratia marcescens, Staphlococcus aureus, Stigmatella aurantiaca, Streptococcus sp. Group B and Streptomyces albus. Both human and mouse lymphocytes were grown in the fermentors. Some of the microorganisms were used for the production of bacteriophages T_4 and lambda, others were used to produce extracellular enzymes. Fermentations were made 48 times in the three 10-liter, 70 times in the 50-liter, 30 times in the 100-liter, 65 times in the 300-liter and twice in the 1100-liter fermentors. Of the 213 fermentations, 35 (16.4%) were contaminated with 20 (9.4%) of these traced to the inocula supplied by the requesting investigator.

Studies were continued with the Division of Bacterial Products, Bureau of Biologics for polysaccharide production by H. influenzae and isolation of the enterotoxin proteins from special strains of E. coli. Spheroplasts were produced for the NHLBI from E. coli using 10-, 50-, and 300-liter fermentors in series. Crude interferon fractions were isolated from cultures of transformed beta lymphocytes stimulated with Newcastle disease virus strain B-1. The Gaulin laboratory homogenizer was used 23 times to rupture cell suspensions of yeast, protozoa, bacteria and mosses.

Large scale processing activities consisted of fractionation of 28 liters of horse Group B meningococcal antiserum to globulin for BoB; isolation of an extra cellular enzyme, Barnase, and its inhibitor, Barstar, from 300 liter cultures of B. amyloliquifaciens; defribination of 50 liter human plasma; dihydrofolate reductase from 94 lbs. beef liver, and preparation of four two-gallon quantities of hydroxylapatite in the 50-gallon Pfaudler glass-lined reactor. Various plants and animal tissues were either dried and/or ground in the large oven, mills and related equipment and solutions were centrifuged, concentrated or lyophilized such as 72 liters Kilham rat virus, 240 liters Bordetella pertussis, 140 liters S 49 mouse lymphoma, 18 liters N. meningitidis, 90 liters potato extract and several 10-15 liter solutions of plant extracts.

Publications: None.

ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMISTRY AND METABOLISM

A. The Role of Hepatic Plasma Membranes in the Recognition and Catabolism of Circulating Glycoproteins. Continuing investigation into the mechanism whereby hepatic membranes bind and transport glycoproteins into the parenchymal cells may be summarized as follows:

1. Examination of the topological distribution of the hepatic binding protein for desialylated glycoproteins revealed this receptor to be on the external, or cytosolic, surface of the lysosomal membranes. Together with the observation that the half life of the plasma membrane receptor was invariant under conditions of prolonged endocytosis of specific ligand, these data have been interpreted as indicative of a recycling mechanism whereby the receptor is spared destruction in the lysosomes and remains available for subsequent re-insertion into the hepatocyte plasma membrane as part of a continuing process. Further evidence in support of this hypothesis has been forthcoming from experiments on isolated lysosomes and on intact hepatocytes. Turnover studies in which protein synthesis was totally blocked by cycloheximide has yielded convincing evidence for a membrane recycling mechanism.

In an alternate approach, the translocation of the receptor from the external surface of the plasma membrane to the cytosolic face of the lysosomes has been studied in a model system wherein isolated hepatocytes imbibe latex particles. A time-dependent eversion of the receptor protein from the inner to the outer surface of the limiting membrane has been shown to be accompanied by fusion of the phageosomes with endogenous lysosomes. Examination of the mechanism of membrane fusion and the relation of these processes to the apparent translocation of cell surface glycoproteins is in progress.

2. In addition to the above problems, an examination of the role of specific membrane receptor for the binding and uptake of the haptoglobin-hemoglobin complex has been undertaken. Studies, in vivo, have demonstrated a time-dependent clearance of the complex from the mammalian circulation with the major locus of sequestration being in the parenchymal cells of the liver. The receptor appears to be extremely labile and has not been successfully demonstrated in isolated liver cells nor, indeed, in a perfused liver. Despite this difficulty, preliminary evidence has been obtained to indicate a role for the carbohydrate moiety of haptoglobin in the recognition event. Initial attempts at solubilization of the presumed receptor have been encouraging in that a soluble extract has been isolated from mammalian liver in which ligand-specific binding has been demonstrated.

B. The Formation of the Yeast Primary Septum.

1. Localization of Chitin on the Yeast Cell Wall. In order to establish the precise topology of chitin on yeast septa and cell walls, ultrastructural and radiochemical methods were used. The former consisted

in determining by electron and fluorescence microscopy the distribution of chitin, as revealed by wheat germ agglutinin or chitinase bound to gold particles (electron microscopy) or to fluorescein isothiocyanate (fluorescence microscopy). In addition, chitin was specifically labeled by growing yeast in the presence of [^{14}C]glucosamine. By subsequent degradation of cell walls with different enzymes and analysis of the radioactive products, it was possible to quantify the results of ultrastructural observations. The conclusions from these studies are as follows: 1) chitin is uniformly distributed on primary septa, i. e., the whole primary septum appears to consist of chitin; 2) most of the cell wall chitin (> 90%) is in the septa, as was indicated but not proven by previous observations. 3) A small amount of chitin (< 10% of the total) is uniformly distributed on the remainder of the cell wall. 4) Both in septa and lateral cell walls chitin appears to be strongly associated with β-(1→6)-glucan. Covalent linkages between the two polysaccharides were not found but could not be excluded.

2. Activation of the chitin synthetase zymogen. The recent discovery, by D. H. Wolf (Wolf, D. H. and Ehmann, C., FEBS Letters 92, 121-124 (1978)) and by E. Jones (Zubenko, G. S., Mitchell, A. P. and Jones, E. W., Proc. Natl. Acad. Sci. USA 76, 2395-2399 (1979)), of yeast mutants that lack proteinase B and are still able to synthesize chitin in vivo, suggested that the postulated function of proteinase B in the activation of chitin synthetase was in error. Therefore efforts have been started to identify another mechanism for chitin synthetase zymogen activation, with the use of proteinase B-deficient mutants, kindly provided by Dr. Jones. Preliminary experiments have shown that it is possible to obtain active chitin synthetase by subjecting intact cells of the mutants to treatments that cause permeabilization. Attempts are now being made to obtain the activation in cell-free extracts.

3. An endochitinase from wheat germ. The study of an endochitinase from wheat germ has been completed. A paper has been published in the Journal of Biological Chemistry. This enzyme has been used, after attachment to gold particles or to fluorescein isothiocyanate, for ultrastructural studies of chitin distribution in the yeast cell wall, that are summarized above.

4. β-(1→3)-glucan synthetase. Studies of this important enzyme, that catalyzes the biosynthesis of the major polysaccharide of the yeast cell wall, have continued. A major finding was that not only ATP, but also GTP could act as an activator of the enzyme. Whereas activation of the synthetase by ATP requires time and a relatively high temperature, the effect of GTP occurs at 0° and is very fast. It can also be mimicked by GTP analogs, whose γ-phosphate cannot be used for phosphorylation because of the chemical groups intercalated between β and γ phosphate residues. It has been concluded that GTP and its analogs act by simple binding to a regulatory site either on the enzyme or on a subunit thereof, whereas ATP probably participates in an enzymatic reaction. The substrate for this reaction, appears to be a substance of small molecular weight, loosely attached to the particulate glucan synthetase. ATP would transform this factor into a

tightly-bound activator of the synthetase. The factor could not yet be identified because of the extremely small amounts available, but it may be related to an activator of the synthetase that was previously found in boiled yeast extracts in this laboratory. Studies on the activator had to be interrupted, because of the new findings about GTP and its function, but are again being pursued at this time.

It has also been found that glucan synthetase can be isolated in a highly active state, by increasing the concentration of the EDTA in buffers used in the preparation of the particulate enzyme. Incubation of this preparation with Mg^{++} leads to inactivation, which can, however, be reversed by addition of either ATP or GTP. It appears, therefore, that the synthetase can be reversibly inactivated and reactivated. This process may be of great importance in the regulation of cell wall synthetase in vivo.

It has now been shown that two enzymes involved in the formation of structural polysaccharides, chitin and glucan synthetase, are subject to a complex regulatory system. It is hoped that these findings will lead to a deeper understanding of processes involved in morphogenesis in other biological systems.

C. The Function of the Bacterial Outer Membrane Components.

1. Relationship of protein E to protein II*. The E. coli outer membrane contains 3-4 proteins which are present in a large number of copies per cell (ca. 10^5/cell). These proteins are distinct in that they have different apparent molecular weights, unique patterns of peptides are generated by cyanogen bromide cleavage, and each protein serves as a receptor for specific bacteriophages. They have shown that purified protein E efficiently inactivates not only bacteriophages that use protein E as their receptor, but also bacteriophages K3 and TuII* that use protein II* as a receptor. Since protein II* also serves in the F^- strain to hold mating pairs together in Hfr x F^- conjugation, they also examined the ability of protein E to restore bacteriophage sensitivity and mating ability in E. coli ompA mutants missing protein II*. The presence of protein E could not restore these functions. This indicates that a putative common region shared by proteins E and II* and responsible for the specific in vitro inactivation of bacteriophages is not accessible in vivo.

2. Relationship of protein E to protein Ia and Ib. Drs. Foulds and Chai have found that proteins E, Ia and Ib are all closely associated with the peptidoglycan larger of the cell envelope, have similar amino acid compositions, identical N-terminal amino acids and function to form pores in the outer membrane that allow the diffusion of small hydrophilic molecules across the outer membrane barrier. In addition, they have demonstrated that protein E contains all the antigenic determinants found on proteins Ia and Ib and additional ones as well. Protein E is larger than proteins Ia and Ib.

3. A working hypothesis. The synthesis of proteins E, Ia, Ib and II* is determined by unique genes on the E. coli chromosome. They have identified the genes responsible for the synthesis of proteins E, Ia and II*. They hypothesize that these genes have evolved by multiple duplications followed by separate evolutions. This hypothesis can be tested in three ways: a) cloning the genes responsible and doing hybridization and sequence studies of cloned DNA: b) isolation and sequence determination of specific messenger RNA molecules; c) sequence analysis of individual proteins.

4. Collaborative studies. a) with M. Zasloff, the lungs of cystic fibrosis patients invariably become infected with Pseudomonas. This infection is usually initiated by a rough strain of Pseudomonas but later only mucoid strains are isolated. A comparison of the cell envelopes and especially the outer membranes prepared from isogenic rough and smooth strains isolated from cystic fibrosis patients has been initiated. b) with J. Robbins, pathogenic E. coli K-1 strains are able to infect newborn mice. They have initiated a study to determine the role of certain outer membrane component proteins and lipopolysaccharides in pathogenicity. In addition the isolation of a variety of mutants which may be altered in the composition of the cell envelope has begun.

D. Hormone Dependent Differentiation of Mammary Gland.

1. 17β-estradiol (E_2) has long been recognized as one of several hormones required for the growth of mammary epithelium. However, it has not heretofore been implicated in mammary epithelial differentiation. The epithelium derived from normal animals can, in fact, differentiate (i.e. make casein and α-lactalbumin) in chemically defined medium in the presence of insulin (I), hydrocortisone (F) and prolactin (P). Under the standardized conditions employed, exogeneous E_2, does not affect the synthesis of the milk proteins. Recently, it was observed that E_2 (1 ng/ml) can augment lactose synthetase activity and casein synthesis in mammary explants in the presence of I, F, and P (50 ng/ml), if triiodothyronine (0.65 ng/ml) is added. Sex steroid specificity was supported by the observations that the E_2-effects are blocked by the anti-estrogen, Nafoxidine, and that 17α-estradiol was inactive; however, estrone and diethylstilbestrol were as active as E_2. Testosterone and progesterone were inhibitory. Since E_2 did not alter the amount of epithelial DNA, its effects represent an increase in biological activities per cell. This is the first demonstration of a differentiative effect of E_2 directly on mammary tissue.

2. Glucocorticoids have been considered to be "permissive" hormones in relation to mammary epithelial differentiation. In the presence of insulin they permit the formation of rough endoplasmic reticulum, organelles required for the formation of the secretory proteins, casein and α-lactalbumin. They also appear to enhance the synthesis of casein mRNA under the primary impetus of prolactin. It has been assumed that α-lactalbumin mRNA, too, is formed under the primary influence of prolactin. Recent experiments have confirmed earlier observations in regard to casein induction: casein emerges in the presence of I, F and P,

but not in the presence of I and F alone. α-Lactalbumin also emerges rather promptly in the presence of I, F and P. Surprisingly however, after a 2-day lag period, it also emerges in the presence of I and F, but in the absence of exogenous P. This is the first indication that relationships between α-lactalbumin and hormones may be different from those for casein. This has important implications in regard to lactogenesis.

E. Polyamines in the Hormone-Dependent Development of Mammary Gland.

1. Earlier studies of the action of glucocorticoid in this system have shown that the steroid, in the presence of insulin, facilitates sustained accumulation of rough endoplasmic reticulum (RER), a key site for the formation of milk-proteins. Our present studies show that such action of glucocorticoid involves augmentation of the biosynthesis of phosphatidylcholine, a major phospholipid component of RER of mammary cells. Further, the increased formation of phosphatidylcholine has been shown to be effected through the glucocorticoid stimulation of the activity of choline kinase, which is involved in the formation of the precursors for the membrane components. The hormonal stimulation of choline kinase activity appears to involve the action of spermidine, a polyamine which accumulates in the cells under the influence of cortisol and mimicks the effect of cortisol on milk-protein synthesis in cultured explants.

2. Previous studies suggested that insulin may exert its effect via changes in plasma membrane structure and intracellular cation concentrations. To gain further insight into the possible role of cations in the hormonal regulation of DNA synthesis in mammary epithelium, we have studied the effect of various inorganic cations on the growth and development of mammary cells in organ culture. Results of these experiments show that lithium ion at concentrations between 2 to 20 mM simulated the stimulatory effects of insulin on the uptake of α-aminoisobutyric acid, synthesis of RNA and DNA, and cell multiplication in mouse mammary gland explants in a chemically defined synthetic medium. Other monovalent cations were virtually ineffective. In most instances, the stimulatory effect of lithium ion is somewhat smaller than and additive to that of insulin. Lithium ion, however, is incapable of substituting for the action of insulin in augmenting milk protein synthesis in mammary explants cultured with other lactogenic hormones, prolactin and glucocorticoid. The observed similarities of the responses of mammary cells to lithium and insulin suggest the possible importance of cation(s) in the regulation of mammary cell proliferation which may be a common basis for the action of the two agents. On the other hand, the observed inability of lithium to mimic the lactogenic effect of insulin indicates a specific function of the hormone in the functional differentiation of mammary cells.

F. Differentiation of Lymphoid Cells.

1. In earlier studies we devised a procedure for iodination of the lipid A moiety of LPS and then utilized the product to establish its preferential binding by B lymphocyte-enriched populations. The present work made use of immunofluorescence (to label B cells) in combination with

autoradiography to label cells that bound [^{125}I]-lipid A. It was observed that 20-30 percent of B cells exhibited binding of lipid A, whereas virtually no binding was observed in thymocyte preparations or in θ bearing cells of spleen. B cells are the predominant binding populations in spleen. Indeed, splenocytes other than B lymphocytes ordinarily exhibit marginal binding when detected. The percentage of cells binding lipid A is in accord with the percentage of cells known to exhibit a mitogenic response. Mouse strains genetically defective in regard to a mitogenic response to lipid A nonetheless bind [^{125}I]-lipid A as well as responder strains indicating that the genetic defect is related to some phenomena other than binding, per se.

2. Concanavalin A, added to freshly isolated rabbit thymocytes, markedly enhanced the extracellular appearance of non-immunoglobulin proteins. Time course studies revealed that the onset of enhancement occurred virtually without delay. The effect appeared to be restricted only to certain of the thymus-derived cells because thymocytes obtained from rabbits treated with hydrocortisone, as well as splenocytes derived from untreated rabbits essentially did not exhibit the enhancement. Stimulation by concanavalin A was specific in that pokeweek mitogen and lipopolysaccharide were without effect and also in that α-methyl-mannoside, but not galactose, abrogated the concanavalin A-mediated enhancement. Experiments with mouse thymocytes demonstrated that the cells which responded to concanavalin A were primarily cells that bear the θ-antigen on their surface (T-cells).

The lipid A moiety of lipopolysaccharide induced freshly isolated bone marrow cells to exhibit enhanced release of [^{3}H]-proteins other than [^{3}H]-immunoglobulins. Such stimulation by lipopolysaccharide was not observed with cell suspensions from spleen, thymus, appendix, peritoneal exudate (macrophage), whole blood, reticulocyte-enriched blood, lymph nodes, liver, testis and the buffy coat of blood. The extracellular appearance of [^{3}H]-proteins was shown to be due to secretion by excluding other alternatives. Thus, the rate of cell death and/or leakage of cellular contents, as well as the rate of shedding of surface membrane protein, was unaffected by lipopolysaccharide. Secretion was selective as judged by the finding that the rate of immunoglobulin release by bone-marrow cells was not enhanced by lipopolysaccharide. The secretory product does not appear to be related to a lymphokine released by macrophage cells and similarily does not appear to have an obligate relationship to the process of mitogenesis. Mouse strain C_3H/HeJ (lipid A non-responder) did not exhibit the enhanced secretion observed by using bone marrow cells of strain C_3H/HeN (lipid A responder).

G. Enzymatic Basis of Detoxication.

1. Recent work with another enzyme of detoxication, thiol S-methyltransferase, has resulted in a 10,000-fold purification from rat liver to yield a homogeneous protein. The enzyme appears to be a microsomal one but can be solubilized by a cycle of freezing and thawing. The purified soluble protein is active with all the lipophilic thiols that have been tested but not with the polar, physiological thiols, i.e. cysteine and GSH.

The enzyme displays remarkable kinetic behavior in which it participates in a single, rapid cycle of reaction to form one mole of products; one product, S-adenosylhomocysteine, is inhibitory and its release from the enzyme is sufficiently rate-limiting that the subsequent rate of reaction is appreciably slower.

2. The major study in progress is that of a group of enzymes in which 3'-phosphoadenosine 5'-phosphosulfate is effective in sulfating phenols, alcohols, sterols, hydroxamic acids and aromatic amines. Six of these enzymes are now being purified and characterized.

H. Biochemical Abnormalization Underlying Human Cystinosis.

1. Previous work has indicated that cultured cells from patients with I-cell disease (mucolipidosis II), an abnormality not known to be related to cystinosis, are characterized by excessively high intracellular levels of free cystine. These initial observations have been extended to additional cell lines, with the following overall results: of fourteen I-cell lines examined, twelve have been found with abnormally high cystine content (range: 2-30 nanomoles "half-cystine" per mg protein; normal cells have less than 0.5 nanomoles half-cystine/mg); two I-cell lines were normal with respect to intracellular cystine. These abnormal levels in I-cells are comparable in magnitude to those seen in cells from cystinotic patients. In contrast to these results, nine of eleven cell lines from patients with mucolipidosis III (pseudo-Hurler polydystrophy), a benign form of I-cell disease, possessed essentially normal levels of cystine, while two of these lines showed marginally elevated levels (1-2 nanomoles half-cystine/mg protein). Cell lines from patients with other lysosomal storage disorders (e.g., Hurler and Hunter diseases; mucolipidosis I) contained normal levels of cystine. Detailed studies of the subcellular distribution of cystine in cultured cystinotic and I-cells indicated that in both types of cells the excess free cystine is associated predominately with the lysosomal fraction.

I. Studies on Naturally Occurring Sulfur Nucleotides.

1. Identification of the λ-induced thionucleotide has not been completed, but it is known to be a thiolated, methylated purine bearing a positive charge. On the basis of its chemical and chromatographic properties and the results of radioactive labeling experiments, it may be 7-methyl-6-thioguanosine. Synthesis of this material has been reported in the literature, but thus far efforts to duplicate the synthesis both here and in the original investigator's laboratory have failed. Methods for the large-scale isolation of this thionucleotide have been developed, and ultraviolet spectra and data from nmr and mass spectroscopy will aid in positive identification.

2. The major species of λ-modified tRNA, purified about 300-fold could not be demonstrated to have amino acid accepting activity, although all of the synthetases were present, and other tRNA peaks from the final column were active. It is known that 7-methyl guanine can easily be split off of the tRNA chain, leaving behind the fragile ribose-phosphate backbone of the tRNA, and it may be that such a session has occurred with the thionucleotide.

J. Thermodynamics and Kinetic Studies of Protein Structure and Enzymatic Mechanisms

1. Kinetic studies on the unfolding and refolding of swine pepsinogen by urea have shown at least two intermediates between the random coil and the native protein. On removal of urea, the peptide chain can fold very rapidly to a form having native secondary structure. This undergoes a slow transition, as shown by changes in the absorbance of the protein, to a second intermediate, which then changes to the native form, which can be activated to pepsin. This last step shows no other detectable changes in the protein's properties, suggesting it to be a very minor change. Variations in experimental conditions indicate that the slow reaction is the same as that in the previously studied alkaline inactivation of pepsinogen, which involves cleavage of an ionic or salt bridge in the zymogen.

2. Similar studies on the urea denaturation of pepsin have shown the presence of a stable intermediate form of the enzyme which has extensive secondary structure. These studies indicated that removal of 44 amino acids from the zymogen to form the active enzyme reduced the stability of the molecule by 3 Kcal $mole^{-1}$.

K. Enzymes of Carbohydrate Metabolism in Invertebrates and Vertebrates.

1. Studies of some of the enzymes of carbohydrate metabolism in marine invertebrates have been continued. Methods are being developed for the separation of hexokinase isozymes and sugar dehydrogenases of Limulus polyphemus by means of polyacrylamide gel electrophoresis and DEAE-ion exchange chromatography. Since hexose and pentose dehydrogenases appear to be quantitatively more important in the hepatopancreas or midgut glands of those invertebrates which we have studied, e.g. Limulus, starfish, squid, lobster and clams, than in the livers of vertebrates, we have been trying to evaluate the possible role of these enzyme in the initial stages of the metabolism of sugars in invertebrates. The turnover of muscle glycogen in Limulus has been studied using $U^{14}C$ glucose as a label. Muscle glycogen of Limuli appears to be replaced at a relatively slow rate in well fed animals, the maximum isotope incorporation after a single injection of the labeled compound being reached after about 10 hours and falling to about 50% of the maximum by 48 hours.

2. Changes in the activities and latencies of four of the key enzymes of the endoplasmic reticular membranes of rat liver and kidney during the neonatal and early weaning periods of rapid membrane proliferation have been studied. The distribution of the phospholipid dependent glucose-6-phosphatase activity between rough and smooth microsomes as a function of age was found to be very different from that of the other enzymes studied. The activities of *sn*-glycerol-3-phosphate acyltransferase, NADPH-cytochrome c reductase and nucleoside diphosphatase remain parallel in the rough and smooth microsomes during postnatal changes in the quantity of enzyme, the rough membranes showing somewhat more activity. This is in accord with a picture of enzymes, newly synthesized on ribosome-bearing membranes, being rapidly distributed to the smooth membranes. On the

other hand, glucose-6-phosphatase showed non-parallel changes with age in the rough and smooth membranes, the well-established neonatal overshoot in activity being almost entirely due to changes in the enzyme in the rough ER. The enzyme activity in the RER reaches a maximum and then decreases after day 2 while that in the smooth membrane is still slowly increasing. It appears that lateral diffusion of glucose-6-phosphatase along the membrane is proceeding at a slower rate than is its replacement by synthesis and degradation within the developing membranes of the neonatal liver.

3. The latencies (i.e., fraction of total activity which is not expressed before activation) of two catalytic activities associated with the same enzyme, glucose-6-phosphatase, were found to show strikingly different age related changes in liver but not in kidney. The latency of PP_i-glucose phosphotransferase activity reaches high levels (60-80% latent) soon after birth and remains high throughout life, while the latency of glucose-6-P phosphohydrolase, very high immediately after birth, decreases with age, reaching the adult latency level of about 20% by 10 days. This is a novel observation of different developmental changes in latency for two functions of a single membrane bound enzyme.

L. The Role of Free and Lipid-Bound Inositol in Brain and Cell Membrane.

1. L-*myo*inositol 1-phosphate synthase (EC 5.5.1.4) is maximally active at pH 7.7. Neither fatt acids nor phospholipids affect the activity, an observation of interest in connection with the previously found (see 1977 annual report) inhibition of glycolysis by these agents in brain, another active site of inositol synthesis. The proposed reaction sequence for which there is indirect evidence is an intramolecular aldol condensation cyclizing 5-ketoglucose 6-phosphate to inosose-2 1-phosphate. Classical aldolases fall into two categories, CLass I, Schiff base-forming, and Class II, metal-requiring. To test for Schiff base formation the enzyme was reduced with $NaBH_4$ in the presence of ^{14}C-glucose 6-phosphate; there was neither inhibition of the enzyme nor binding of label, indicating the absence of Schiff base formation. The enzyme was likewise unaffected by 100 mM EDTA, suggesting no metal requirement. The enzyme thus fits into neither category of aldolase. Cations, however, have a profound effect on the activity. With $Tris^+$ (50 mM) and NAD^+ (1 mM) the sole cations (basal conditions) 1 mM K^+ or NH_4^+ stimulated 275 and 400%, resp. Zn^{2+}, Cd^{2+}, and Cu^{2+} (1 mM) abolished activity but the effect of Zn^{2+} and Cd^{2+} was completely reversed by 10 mM EDTA. The effect of Cu^{2+} was irreversible even with 50 mM EDTA. Monovalent cations in the order $Tris^+ < Na^+ < K^+ < Li^+ < NH_4^+$ increased the affinity (decreased the K_m) up to 15-fold for glucose 6-phosphate and 10-fold for NAD^+. The effect of Li^+ on V_{max}, however, was anomalous. Whereas the other cations increased V_{max} in the order $Tris^+ < Na^+ < K^+ < NH_4^+$ up to 250% for glucose 6-phosphate and 440 for NAD^+, Li^+ decreased V_{max} 32% for glucose 6-phosphate with no effect on V_{max} for NAD^+. We conclude that binding of substrate and cofactor are sensitive to the ionic environment.

M. Biochemical Lesions in Genetic Mucopolysaccharidoses.

1. The disulfated ^{35}S-labeled methyl glucosaminide disulfide prepared by direct sulfation was found to comprise two major components, in a ratio of approximately 7:3, the larger of which was unaffected by the enzyme, whereas the smaller was totally hydrolyzed. To establish the structure of the active substrate, unequivocal synthesis of the N-sulfated α-methyl glucosaminide-6-sulfate and the corresponding -4- and -3- sulfates was undertaken. The -6- and -4- sulfates have been prepared via s series of carbobenzoxy, triphenyl methyl and acetyl blocked crystalline intermediates. The structure of the 6-sulfated derivative has been confirmed by elementary and NMR spectral analysis. Neither of these compounds corresponds to the ^{35}S-labeled substrate prepared by direct sulfation. We may infer that the active substrate is most likely the N-sulfated-α-methylglucosaminide-3-sulfate.

2. A simple column technique has been developed to assay the enzyme replacing the previous procedure based on electrophoresis. The enzyme is maximally active at pH 6.3 and is inhibited by chloride and sulfate ions. No significant activity has been detected in extracts of cultured fibroblasts.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 17001-13 LBM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Role of Hepatic Plasma Membranes in the Turnover of Circulating Glycoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gilbert Ashwell	Chief, Lab. of Biochem. & Metab.	LBM	NIAMDD
OTHER:	Jean Hickman	Research Chemist	LBM	NIAMDD
	William Pricer	Research Chemist	LBM	NIAMDD
	Mark Lowe	Staff Fellow	LBM	NIAMDD
	Joseph Harford	Guest Worker	LBM	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies on the subcellular loci of a specific hepatic membrane protein responsible for the recognition and catabolism of desialylated serum glycoproteins have established the presence of this receptor on the internal (luminal) surface of the golgi complex and the smooth membranes in contrast to its location on the external (cytosolic) surface of the lysosomes. The latter finding has been correlated with the recycling of this receptor under conditions of maximal catabolism of the receptor-asialoglycoprotein complex. Further insight into the mechanism of this translocation of the membrane protein has been obtained in a model system utilizing the rapid pinocytosis of latex particles by isolated hepatocytes.

PHS-6040
(Rev. 10-76)

Project Description:

The role of Hepatic Plasma Membranes in The Recognition and Catabolism of Circulating Glycoproteins. Continuing investigation into the mechanism whereby hepatic membranes bind and transport glycoproteins into the parenchymal cells may be summarized as follows:

1) Examination of the topological distribution of the hepatic binding protein for desialylated glycoproteins revealed this receptor to be on the external, or cytosolic, surface of the lysosomal membranes. Together with the observation that the half life of the plasma membrane receptor was invariant under conditions of prolonged endocytosis of specific ligand, these data have been interpreted as indicative of a recycling mechanism whereby the recepto is spared destruction in the lysosomes and remains available for subsequent re-insertion into the hepatocyte plasma membrane as part of a continuing process. Further evidence in support of this hypothesis has been forthcoming from experiments on isolated lysosomes and on intact hepatocytes. Turnover studies in which protein synthesis was totally blocked by cycloheximide has yielded convincing evidence for a membrane recycling mechanism.

In an alternate approach, the translocation of the receptor from the external surface of the plasma membrane to the cytosolic face of the lysosomes has been studied in a model system wherein isolated hepatocytes imbibe latex particles. A time-dependent eversion of the receptor protein from the inner to the outer surface of the limiting membrane has been shown to be accompanied by fusion of the phageosomes with endogenous lysosomes. Examination of the mechanism of membrane fusion and the relation of these processes to the apparent translocation of cell surface glycoproteins is in progress.

2) In addition to the above problems, an examination of the role of specific membrane receptors for the binding and uptake of the haptoglobin-hemoglobin complex has been undertaken. Studies, in vivo, have demonstrated a time-dependent clearance of the complex from the mammalian circulation with the major locus of sequestration being in the parenchymal cells of the liver. The receptor appears to be extremely labile and has not been successfully demonstrated in isolated liver cells nor, indeed, in a perfused liver. Despite this difficulty, preliminary evidence has been obtained to indicate a role for the carbohydrate moiety of haptoglobin in the recognition event. Initial attempts at solubilization of the presumed receptor have been encouraging in that a soluble extract has been isolated from mammalian liver in which ligand-specific binding has been demonstrated.

Proposed Course of Project:

1) It is proposed to further document the orientation of the membrane protein on the lysosomes by electron microscopic examination of the affinity bound particles coupled to a monospecific antibody.

2) An attempt will be made to study the mechanism of plasma membrane endocytosis utilizing fluorescence microscopy of the appropriately labeled antibody to the specific receptor on isolated hepotocytes. Model experiments on polyethylene glycol mediated cell fusion will be undertaken.

3) Studies on the hepatic receptor for the haptoglobin-hemoglobin complex will be continued with the aim of isolating the binding protein in purified form as a prelude to the determination of the specific recognition signal which initiates clearance from the circulation.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 17002-09 LBM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Enzymatic Basis of Detoxication

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William B. Jakoby	Chief, Section on Enzymes and Cellular Biochemistry	LBM NIAMDD
OTHER:	Ellen Sue Lyon	Staff Fellow	LBM NIAMDD
	Ronald D. Sekura	Staff Fellow	LBM NIAMDD
	Jun-Lan Wang	Guest Worker	LBM NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIAMDD,NIH, Bethesda, Maryland 20

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A major design consideration for enzymes active in the detoxication of foreign compounds, appears to be a broad specificity for primarily lipophibic molecules. The glutathione transferases are a good example in that they catalyze any reaction in which glutathione serves as a nucleophile if the second substrate is bound to the protein; act as binding and storage proteins intracellularly for bilirubin among thousands of other ligands; and form irreversible covalent complexes with very reactive electrophiles including carcinogens. Thiol S-methyltransferase, recently obtained in homogeneous form, is another example of a detoxicating enzyme active in transferring a methyl group to lipophilic mercaptans. A similar situation obtains with phenol and sterol sulfotransferases, enzymes which are now also available as homogeneous proteins.

PHS-6040
(Rev. 10-76)

Project Description:

Methods Employed - An effort is being made to bring our knowledge of detoxication processes to the level at which they may be discussed in terms of characterized specific enzymes of high purity. This point has now been reached for the glutathione transferases, a group of enzymes of overlapping and broad specificity for compounds bearing an electrophilic atom. They catalyze the reaction of GSH, acting as nucleophile, with any of the tens of thousands of lipophilic compounds that can bind to the protein and are sufficiently electrophilic.

Major Findings - Recent work with another enzyme of detoxication, thiol S-methyltransferase, has resulted in a 10,000-fold purification from rat liver to yield a homogeneous protein. The enzyme appears to be a microsomal one but can be solubilized by a cycle of freezing and thawing. The purified soluble protein is active with all the lipophilic thiols that have been tested but not with the polar, physiological thiols, i.e. cysteine and GSH. The enzyme displays remarkable kinetic behavior in which it participates in a single, rapid cycle of reaction to form one mole of products; one product, S-adenosylhomocysteine, is inhibitory and its release from the enzyme is sufficiently rate-limiting that the subsequent rate of reaction is appreciably slower.

Significance - The major study in progress is that of a group of enzymes in which 3'-phosphoadenosine 5'-phosphosulfate is effective in sulfating phenols, alcohols, sterols, hydroxamic acids and aromatic amines. Six of these enzymes are now being purified and characterized.

Proposed Course - Work with the conjugating enzymes of liver is intended to delineate the means by which we are able to survive in the presence of the many noxious and toxic compounds to which we are exposed. The investigation of the above noted projects will be continued with particular emphasis on the enzymes responsible for sulfation.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 17003-12 LBM

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Polysaccharides in Morphogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Enrico Cabib	Research Chemist	LBM	NIAMDD
OTHER:	Eleanor Shematek	Staff Fellow	LBM	NIAMDD
	Martin Slater	Staff Fellow	LBM	NIAMDD
	Itzhack Polacheck	Guest Worker	LBM	NIAMDD
	Julieta Correa	Guest Worker	LBM	NIAMDD

COOPERATING UNITS (if any)

Blair Bowers, LCB, NHLBI

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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SUMMARY OF WORK (200 words or less - underline keywords)

The localization of chitin in yeast cell walls was studied by fluorescence and electron microscopy, with the use of specific markers attached to fluorescein or colloidal gold, respectively. Chitin was also labeled by incorporation of [^{14}C]glucosamine in growing cells. The results confirmed that most of the chitin is in primary septa and that its distribution on the septum is uniform. Some chitin (7-8% of the total) is dispersed over the cell wall.

Further studies on the regulation of yeast β(1→3)glucan synthetase have shown that both ATP and GTP activate the enzyme, but by different mechanisms. GTP seems to act by simply binding to the enzyme or to a regulatory subunit. ATP, on the other hand, would modify, in an enzymatically catalyzed reaction, a small molecular weight compound attached to enzyme, thereby converting it into an activator. The effect appears to be reversible, inasmuch as incubation with Mg^{++} of highly active enzyme, prepared in the presence of 5-10 mM EDTA, leads to an inactivation, which can be reversed either by ATP or GTP. It is suggested that this reversible activation process may be of great importance for the regulation of cell wall growth in vivo.

mechanism for chitin synthetase zymogen activation, with the use of proteinase B-deficient mutants, kindly provided by Dr. Jones. Preliminary experiments have shown that it is possible to obtain active chitin synthetase by subjecting intact cells of the mutants to treatments that cause permeabilization. Attempts are now being made to obtain the activation in cell-free extracts.

c) An endochitinase from wheat germ. The study of an endochitinase from wheat germ has been completed. A paper has been published in the Journal of Biological Chemistry. This enzyme has been used, after attachment to gold particles or to fluorescein isothiocyanate, for ultrastructural studies of chitin distribution in the yeast cell wall, that are summarized above.

β-(1→3)-glucan synthetase

Studies of this important enzyme, that catalyzes the biosynthesis of the major polysaccharide of the yeast cell wall, have continued. A major finding was that not only ATP, but also GTP could act as an activator of the enzyme. Whereas activation of the synthetase by ATP requires time and a relatively high temperature, the effect of GTP occurs at 0° and is very fast. It can also be mimicked by GTP analogs, whose γ-phosphate cannot be used for phosphorylation because of the chemical groups intercalated between β and γ phosphate residues. It has been concluded that GTP and its analogs act by simple binding to a regulatory site either on the enzyme or on a subunit thereof, whereas ATP probably participates in an enzymatic reaction. The substrate for this reaction, appears to be a substance of small molecular weight, loosely attached to the particulate glucan synthetase. ATP would transform this factor into a tightly-bound activator of the synthetase. The factor could not yet be identified because of the extremely small amounts available, but it may be related to an activator of the synthetase that was previously found in boiled yeast extracts in this laboratory. Studies on the activator had to be interrupted, because of the new findings about GTP and its function, but are again being pursued at this time.

It has also been found that glucan synthetase can be isolated in a highly active state, by increasing the concentration of the EDTA in buffers used in the preparation of the particulate enzyme. Incubation of this preparation with Mg^{++} leads to inactivation, which can, however, be reversed by addition of either ATP or GTP. It appears therefore that the synthetase can be reversibly inactivated and reactivated. This process may be of great importance in the regulation of cell wall synthesis in vivo.

It has now been shown in our laboratory that two enzymes involved in the formation of structural polysaccharides, chitin and glucan synthetase, are subject to a complex regulatory system. It is hoped that these findings will lead to a deeper understanding of processes involved in morphogenesis in other biological systems.

Project Description:

Objectives: The ongoing research attempts to provide an understanding of the molecular mechanisms of morphogenesis. The topics currently under study are the formation of the primary septum of yeast and the biosynthesis of a glucan which is a major structural component of the yeast cell wall.

Methods Employed: The organism used is Saccharomyces cerevisiae, grown in the laboratory. Cells are converted into protoplasts by treatment with snail intestinal juice. Protoplasts are disrupted by osmotic lysis and mechanical shearing to obtain particulate fractions. The latter are further fractionated by centrifugation in density gradients. Enzymes are solubilized with detergents and purified by conventional methods including affinity chromatography on appropriate adsorbents.

Wheat germ agglutinin and purified preparations of different chitinases are attached to colloidal gold, to obtain markers for the localization of chitin in cell walls and septa by electron microscopy. Adducts of the same proteins with fluorescein isothiocyanate are used as chitin markers for fluorescence microscopy.

Major Findings: The Formation of the yeast primary septum.

A) Localization of chitin on the yeast cell wall. In order to establish the precise topology of chitin on yeast septa and cell walls, ultrastructural and radiochemical methods were used. The former consisted in determining by electron and fluorescence microscopy the distribution of chitin, as revealed by wheat germ agglutinin or chitinase bound to gold particles (electron microscopy) or to fluorescein isothiocyanate (fluorescence microscopy). In addition, chitin was specifically labeled by growing yeast in the presence of [^{14}C]glucosamine. By subsequent degradation of cell walls with different enzymes and analysis of the radioactive products, it was possible to quantify the results of ultrastructural observations. The conclusions from these studies are as follows: 1) chitin is uniformly distributed on primary septa, i. e., the whole primary septum appears to consist of chitin; 2) most of the cell wall chitin (> 90%) is in the septa, as was indicated but not proven by previous observations. 3) A small amount of chitin (< 10% of the total) is uniformly distributed on the remainder of the cell wall. 4) Both in septa and lateral cell walls chitin appears to be strongly associated with β-(1→6)-glucan. Covalent linkages between the two polysaccharides were not found but could not be excluded.

A manuscript embodying these studies is in preparation.

B) Activation of the chitin synthetase zymogen. The recent discovery, by D. Wolf (Wolf, D. H. and Ehmann, C., FEBS Letters 92, 121-124 (1978)) and by E. Jones (Zubenko, G. S., Mitchell, A. P. and Jones, E. W., Proc. Natl. Acad. Sci. USA 76, 2395-2399 (1979)), of yeast mutants that lack proteinase B and are still able to synthesize chitin in vivo, suggested that the postulated function of proteinase B in the activation of chitin synthetase was in error. Therefore efforts have been started to identify another

Two papers on the mechanism of action and on the regulatory properties of glucan synthetase have been submitted for publication.

Significance to Bio-Medical Research and the Program of the Institute:

The ongoing and proposed work deals with fundamental processes of differentiation and morphogenesis that occur in all living cells. More knowledge about such basic processes is essential for our understanding of cellular function both in health and in disease.

Proposed Course of Project:

The process of activation of chitin synthetase in proteinase B-deficient yeast mutants will be further investigated.

The initiation of chitin formation during budding of intact yeast cells will be studied with the use of the recently developed ultrastructural markers for chitin.

The purification and chemical analysis of an activator of yeast (β-1→3)-glucan synthetase will be pursued further. Efforts will be made to understand better the complex regulation of this enzyme. In particular, it will be attempted to solubilize the enzyme, in order to analyze the various components of the system.

Publications

Duran, A. and Cabib, E.: Solubilization and partial purification of yeast chitin synthetase. Conformation of the zymogenic nature of the enzyme. J. Biol. Chem. 253: 4419-4425, 1978.

Duran, A., Cabib, E. and Bowers, B.: Chitin synthetase distribution on the yeast plasma membrane. Science 203: 363-365, 1979.

Molano, J., Polacheck, I., Duran, A. and Cabib, E.: An endochitinase from wheat germ. Activity on nascent and preformed chitin. J. Biol. Chem. 254: 4901-4907, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 17004-11 LBM

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Peter McPhie	Research Chemist	LBM	NIAMDD
OTHER:	Faizan Ahmad	Visiting Fellow	LBM	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes & Cellular Biochemistry

INSTITUTE AND LOCATION
NIAMDD, LBM, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	-

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our studies are directed towards the relationship of protein sequence to conformation and enzymic activity. The digestive enzyme, pepsin, is synthesized as an inactive precursor pepsinogen. We are interested in the molecular basis of the proteolytic activity of the enzyme, how this is suppressed in the zymogen and is induced during activation.

PHS-6040
(Rev. 10-76)

Project Description:

Methods Employed - Visible and ultraviolet spectroscopy: Optical rotatory dispersion and circular dichroism; Viscosity; Enzyme assays; Rapid kinetic techniques using stopped flow and T-jump apparatus.

Major Findings - Kinetic studies on the unfolding and refolding of swine pepsinogen by urea have shown at least two intermediates between the random coil and the native protein. On removal of urea, the peptide chain can fold very rapidly to a form having native secondary structure. This undergoes a slow transition, as shown by changes in the absorbance of the protein, to a second intermediate, which then changes to the native form, which can be activated to pepsin. This last step shows no other detectable changes in the proteins properties, suggesting it to be a very minor change. Variations in experimental conditions indicate that the slow reaction is the same as that in the previously studied alkaline inactivation of pepsinogen, which involves cleavage of an ionic or salt bridge in the zymogen.

Similar studies on the urea denaturation of pepsin have shown the presence of a stable intermediate form of the enzyme which has extensive secondary structure. These studies indicated that removal of 44 amino acids from the zymogen to form the active enzyme reduced the stability of the molecule by 3 Kcal $mole^{-1}$.

Proposed Course of Project - It is hoped to delineate more clearly the mechanism by which the structureless polypeptide chain can fold itself into the complex form required for auto activation. Further studies on swine pepsinogen and on similar proteins from other species will aid in this problem.

Previous studies on the reaction of swine pepsinogen with diethyl pyrocarbonate gave rather confusing results. This reagent will be synthesized in radioactive form and its reaction with the zymogen studied in great detail. In particular reaction with other residues, besides histidines, will be looked for.

Publications:

Ahmad, F. and McPhie, P.: The denaturation of covalently inhibited swine pepsin. Int. J. of Peptide and Protein Res. 12: 155-163, 1978.

Ahmad, F. and McPhie, P.: Intrinsic viscosities of randomly coiled glycoproteins. Int. J. Biochem. 9: 647-651, 1978.

Ahmad, F. and McPhie, P.: Spectrophotometric titration of phenolic groups of pepsin. Biochim. Biophys. Acta 537: 247-254, 1978,

McPhie, P.: Alkaline transition of swine pepsinogen. Biophys. Chem. in press.

Publications: (Continued)

Ahmad, F. and McPhie, P.: Characterization of a stable intermediate in the unfolding of DAG-pepsin by urea. Can. J. Biochem. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 17005-14 LBM

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies of Naturally Occurring Sulfur Nucleotides: Identification, Chemistry, Biosynthesis and Biological Significance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marie N. Lipsett	Research Chemist	LBM	NIAMDD
OTHER:	Sankar L. Adhya	Res. Microbiologist	LMBG	NCI

COOPERATING UNITS (if any)

Laboratory of Molecular Biology, BGS, NCI

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIAMDD,NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.1	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (300 words or less - underline keywords)

Identification is proceeding for the new tRNA thionucleotide produced after bacteriophage λ infection of Escherichia coli. Methods have been devised for large-scale isolation of the thionucleotide. The λ site coding for the new thiolating activity has been mapped. An amber mutation at this site can be suppressed by tyrosine, but not by serine or glutamic acid substitutions.

A search for further E. coli mutants deficient in tRNA 4-thiouridine has thus far been fruitless.

OD-c040
Rev. 10-76)

Major Findings and Proposed Course of Work:

1. New thionucleotide (with S. Adhya). Identification of the λ-induced thionucleotide has not been completed, but it is known to be a thiolated, methylated purine bearing a positive charge. On the basis of its chemical and chromatographic properties and the results of radioactive labeling experiments, it may be 7-methyl-6-thioguanosine. Synthesis of this material has been reported in the literature, but thus far efforts to duplicate the synthesis both here and in the original investigator's laboratory have failed.

Methods for the large-scale isolation of this thionucleotide have been developed, and we hope shortly to have ultraviolet spectra and data from nmr and mass spectroscopy to help in positive identification.

The major species of λ-modified tRNA, purified about 300-fold could not be demonstrated to have amino acid accepting activity, although all of the synthetases were present, and other tRNA peaks from the final column were active. It is known that 7-methyl guanine can easily be split off of the tRNA chain, leaving behind the fragile ribose-phosphate backbone of the tRNA, and it may be that such a session has occurred with the thionucleotide.

The mapping of the λ site is primarily the work of Dr. Adhya, although I did the tRNA thionucleotide analysis. On amber mutation in the λ site can be suppressed by Su_{III} (tyrosine) but not by suppressors inserting serine or glutonic acid at this site.

2. The part-time search for additional E. coli mutants showing an absolute lack of 4-thiouridine in the tRNA has produced no further strains.

Publications:

Lipsett, M. N.: The enzymes producing 4-thiouridine in Escherichia coli transfer RNA: Their approximate chromosomal locations and their activities in a 4-thiouridine-deficient mutant. J. Bacteriol. 135: 993-997, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 17006-05 LBM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Function of Bacterial Outer Membrane Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John D. Foulds	Research Chemist	LBM	NIAMDD
OTHER:	Tuu-jyi Chai	Staff Fellow	LBM	NIAMDD
	Joyce Sturgeon	Staff Fellow	LBM	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The outer membrane of Escherichia coli functions as a barrier which allows the passive diffusion of hydrophilic molecules while protecting the cell from detergents such as deoxycholate. We have found that the predominant outer membrane proteins called porin proteins, have a role in this barrier function for when mutant strains missing demonstrable amounts of porin protein were isolated, the uptake of sugars, amino acids and nucleotides was markedly reduced.

We have found that at least 4 of the predominantly outer membrane proteins are functionally and/or immunologically related .

PHS-6040
(Rev. 10-76)

Project Description:

Objectives - The goal of this project is to define the role of individual components (proteins, phospholipids, lipopolysaccharides) in the function of the outer membrane of gram-negative bacteria.

(1) To examine the permeability properties of the outer membrane of Escherichia coli and Pseudomonas strains. Mutants with altered permeability will be isolated and characterized and the phenotypic alterations resulting from the mutation(s) will be identified.

(2) To examine the ability of various types of lipopolysaccharides to promote the inactivation of specific bacteriophages by purified outer membrane proteins.

(3) To reconstitute permeability function and bacteriophage sensitivity by incorporation of purified outer membrane proteins into intact E. coli cells.

Major Findings -

1) Relationship of protein E to protein II*. The E. coli outer membrane contains 3-4 proteins which are present in a large number of copies per cell (ca. 10^5/cell). These proteins are distinct in that they have different apparent molecular weights, unique patterns of peptides are generated by cyanogen bromide cleavage, and each protein serves as a receptor for specific bacteriophages. They have shown that purified protein E efficiently inactivates not only bacteriophages that use protein E as their receptor, but also bacteriophages K3 and TuII that use protein II* as a receptor. Since protein II* also serves in the F⁻ strain to hold mating pairs together in Hfr x F⁻ conjugation, they also examined the ability of protein E to restore bacteriophage sensitivity and mating ability in E. coli ompA mutants missing protein II*. The presence of protein E could not restore these functions. This indicates that a putative common region shared by proteins E and II* and responsible for the specific in vitro inactivation of bacteriophages is not accessible in vivo.

2) Relationship of protein E to protein Ia and Ib. Drs. Foulds and Chai have found that proteins E, Ia and Ib are all closely associated with the peptidoglycan larger of the cell envelope, have similar amino acid compositions, identical N-terminal amino acids and function to form pores in the outer membrane that allow the diffusion of small hydrophilic molecules across the outer membrane barrier. In addition, they have demonstrated that protein E contains all the antigenic determinants found on proteins Ia and Ib and additional ones as well. Protein E is larger than proteins Ia or Ib.

3) A working hypothesis. The synthesis of proteins E, Ia, Ib and II* is determined by unique genes on the E. coli chromosome. They have identified the genes responsible for the synthesis of proteins E, Ia and II*. They hypothesize that these genes have evolved by multiple duplications followed by separate evolutions. This hypothesis can be tested in three ways: a) cloning the genes responsible and doing hybridization and sequence studies of cloned DNA; b) isolation and sequence determination of specific messenger RNA molecules; c) sequence analysis of individual proteins.

4) Collaborative studies. a) with M. Zosloff, the lungs of cystic fibrosis patients invariably become infected with Pseudomonas. This infection is usually initiated by a rough strain of Pseudomonas but later only mucoid strains are isolated. A comparison of the cell envelopes and especially the outer membranes prepared from isogenic rough and smooth strains isolated from cystic fibrosis patients has been initiated. b) with J. Robbins, pathogenic E. coli K-1 strains are able to infect newborn mice. They have initiated a study to determine the role of certain outer membrane components proteins and lipopolysaccharides in pathogenicity. In addition the isolation of a variety of mutants which may be altered in the composition of the cell envelope has begun.

Proposed Course:

1) Reconstitution of membrane function. Bacteriophage TC45 uses protein E as its receptors. E coli strains whose outer membrane do not contain protein E are completely resistant to this bacteriophage. Attempts will be made to incorporate protein E into the outer membrane of viable E coli cells by fusing phospholipid vesicles containing protein E with protein E-negative cells. Cells in which protein E is incorporated will be identified as sensitive to bacteriophage TC45. Bacteriophage which is adsorbed non-specifically will be inactivated using specific antiserum.

2) Isolation of outer membrane protein precursors. It has been demonstrated that many proteins found in the cell envelope are synthesized in the form of a precursor which is later clerved, presumably by a specific endopeptidase. Antibody prepared using purified major outer membrane proteins will be immobilized on a matrix, such as Sepharose, and used to isolate precursor proteins by affinity chromatography. If these efforts are successful, attempts will be made to determine the location and specificity of the endopeptidase which is widely believed to be, in part, responsible for the sequestration of individual proteins in the outer membrane.

Publications:

Chai, T., and Foulds, J.: Isolation and characterization of two bacteriophages which utilize a new Escherichia coli major outer membrane protein as part of their receptor. J. Bacteriol. 135: 164-170, 1978.

Foulds, J., and Chai, T.: Chromosomal location of a gene (nmpA) involved in the expression of a major outer membrane protein in Escherichia coli. J. Bacteriol. 136: 501-506, 1978.

Chai, T., and Foulds, J.: Inactivation of bacteriophages by protein E, a new major membrane protein isolated from an Escherichia coli mutant. J. Bacteriol. 137: 226-233, 1979.

Foulds, J., and Chai, T.: Isolation and characterization of isogenic Escherichia coli K-12 strains with alterations in the level of one or more major outer membrane prtoeins. Can. J. Microbiol. 25: 423-427, 1979.

Chai, T., and Foulds, J.: Isolation and partial characterization of protein E, a major protein found in certain Escherichia coli K-12 mutant strains: Relationship to other outer membrane proteins. J. Bacteriol. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-18000-14 LBM

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Hormone-Dependent Differentiation of Mammary Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Yale J. Topper	Research Chemist, Chief, Section on Intermediary Metabolism	LBM NIAMDD
Other:	Dr. Lakshmanan Sankaran	Staff Fellow	LBM NIAMDD
	Dr. Kevin Nicholas	Visiting Fellow	LBM NIAMDD
	Dr. Kapalavayi Nagaiah	Visiting Associate	LBM NIAMDD
	Dr. Franklyn Bolander Jr.,	Research Associate	LBM NIAMDD

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Intermediary Metabolism

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.0	5	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

178-estradiol can stimulate the synthesis of casein and the activity of lactose synthetase in mammary explants, in the presence of insulin and glucocorticoid, but this effect requires the presence of physiological concentrations of prolactin and thyroid hormone.

The hormonal requirements for the synthesis of α-lactalbumin by mammary explants may be different from those for the synthesis of casein.

HHS-6040
(Rev. 10-76)

Project Description:

Objectives - To study the molecular and cytological phenomena involved in the development of the mammary gland. This includes attempts to understand the mechanism of action of insulin, prolactin, glucocorticoids, estrogen, progesterone and thyroid hormone on this tissue.

Methods Employed -

Mammary gland explants, mammary epithelial cells or mammary cell nuclei are cultured in synthetic media. Synthesis of α-lactalbumin is followed by measuring the formation of lactose in tissue extracts. Synthesis of casein is determined immunochemically, or by electrophoretic isolation of ^{33}P-labeled phosphoproteins. Casein mRNA is assayed by excess c-DNA hybridization. DNA and RNA synthesis are studied using conventional methods.

Major Findings -

A. 17β-estradiol (E_2) has long been recognized as one of several hormones required for the growth of mammary epithelium. However, it has not heretofore been implicated in mammary epithelial differentiation. The epithelium derived from normal animals can, in fact, differentiate (i.e. make casein and α-lactalbumin) in chemically defined medium in the presence of insulin (I), hydrocortisone (F) and Prolactin (P). Under the standardized conditions employed, exogenous E_2, does not affect the synthesis of the milk proteins. Recently, we observed that E_2 (1 ng/ml) can augment lactose synthetase activity and casein synthesis in mammary explants in the presence of I, F, and P (50 ng/ml), if triiodothyronine (0.65 ng/ml) is added. Sex steroid specificity was supported by the observations that the E_2-effects are blocked by the anti-estrogen, Nafoxidine, and that 17 α-estradiol was inactive; however, estrone and diethylstilbestrol were as active as E_2. Testosterone and progesterone were inhibitory. Since E_2 did not alter the amount of epithelial DNA, its effects represent an increase in biological activities per cell. This is the first demonstration of a differentiatiative effect of E_2 directly on mammary tissue.

B. Glucocorticoids have been considered to be "permissive" hormones in relation to mammary epithelial differentiation. In the presence of insulin they permit the formation of rough endoplasmic reticulum, organelles required for the formation of the secretory proteins, casein and α-lactalbumin. They also appear to enhance the synthesis of casein mRNA under the primary impetus of prolactin. It has been assumed that α-lactalbumin mRNA, too, is formed under the primary influence of prolactin. Recent experiments have confirmed earlier observations in regard to casein induction: casein emerges in the presence of I, F and P, but not in the presence of I and F alone. α-lactalbumin also emerges rather promptly in the presence of I, F and P. Surprisingly however, after a 2-day lag period, it also emerges in the presence of I

and F, but in the absence of exogenous P. This is the first indication that relationships between α-lactalbumin and hormones may be different from those for casein. This has important implications in regard to lactogenesis.

Proposed Course of Project -

1. We will attempt to determine whether estrogen is an absolute requirement for the formation of casein and α-lactalbumin by mammary cells.

2. Events which occur during a 2-day lag period in the presence of I and F circumvent the need for exogenous P in regard to the synthesis of α-lactalbumin. We will attempt to delineate these events.

Significance to Biomedical Research and the Program of the Institute -

Hormones are involved in normal growth and development, and are implicated in many disease states. The mammary gland system provides an excellent opportunity to increase our understanding about the ways in which a number of different types of hormones interact with target cells.

Publications:

Freeman, C.S. and Topper, Y.J.: Progesterone is not essential to the differentiative potential of mammary epithelium in the male mouse. Endocrinology 103: 186-191, 1978.

Vonderhaar, B.K., Smith, G.H., Pauley, R.J., Rosen, J.M. and Topper,Y.J.: A difference between mammary epithelial cells from mature virgin and primiparous mice. Cancer Res. 38:4059-4065, 1978.

Freeman, C.S. and Topper, Y.J.: Multiple hormone interactions in the developmental biology of the mammary gland. Physiol. Rev., in press.

Topper, Y.J.:Alterations in steroid-responsiveness during ontogeny of mouse mammary epithelium. presented at Bath-Sheva Seminar Israel, 1978.

Topper, Y.J. and Bolander, F., Jr.: Stimulation of lactose synthetase activity and casein synthesis in mouse mammary explants by estradiol. Endocrinology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM18001-05 LBM

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Role of Free and Lipid-bound Inositol in Brain and Cell Membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Frank Eisenberg Jr.,	Research Chemist	LBM NIAMDD
Other:	Dr. Toshio Maeda	Visiting Fellow	LBM NIAMDD

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Intermediary Metabolism

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Further properties and the mechanism of action of homogeneous inositol 1-phosphate synthase (EC 5.5.1.4) from rat testis have been examined. The enzyme is maximally active at pH 7.7. Relative to basal activity measured in the presence of Tris+ (50 mM) and NAD+ (1 mM) as sole cations, the addition of 1 mM K^+ or NH_4^+ stimulates the activity 275 and 400%, respectively. Both cations decrease K_m and increase V_{max} markedly for both substrate (glucose 6-phosphate) and cofactor (NAD^+). Li^+ also decreases K_m but anomalously decreases V_{max} for glucose 6-phosphate. Heavy metals inhibit completely, Zn^{2+} and Cd^{2+} reversibly and Cu^{2+} irreversibly. EDTA up to 100 mM has no effect on basal activity suggesting no metal requirement and eliminating the enzyme as a Class II aldolase. Failure of $NaBH_4$ reduction to inactivate the enzyme and to bind label in the presence of labeled substrate rules out a Schiff base mechanism eliminating the enzyme as a Class I aldolase.

PHS-6040
(Rev. 10-76)

Project Description:

Objective - Properties and mechanism of action of L-*myo*inositol 1-phosphate synthase.

Methods - L-*myo*inositol 1-phosphate synthase (EC 5.5.1.4) was isolated from rat testis, a rich source of the enzyme, and purified to homogeneity by methods described previously (see 1978 annual report). The activity of the enzyme was assayed by incubation with glucose 6-phosphate (substrate) and NAD^+ (cofactor) followed by a second incubation with periodate which liberates inorganic phosphate from inositol 1-phosphate (product) under conditions in which glucose 6-phosphate is relatively stable. Inorganic phosphate is then assayed by conventional procedures.

Major Findings - The enzyme is maximally active at pH 7.7. Neither fatty acids nor phospholipids affect the activity, an observation of interest in connection with the previously found (see 1977 annual report) inhibition of glycolysis by these agents in brain, another active site of inositol synthesis. The proposed reaction sequence for which there is indirect evidence is an intramolecular aldol condensation cyclizing 5-ketoglucose 6-phosphate to inosose-2 1-phosphate. Classical aldolases fall into two categories, Class I, Schiff base-forming, and Class II, metal-requiring. To test for Schiff base formation the enzyme was reduced with $NaBH_4$ in the presence of ^{14}C-glucose 6-phosphate; there was neither inhibition of the enzyme nor binding of label, indicating the absence of Schiff base formation. The enzyme was likewise unaffected by 100 mM EDTA, suggesting no metal requirement. The enzyme thus fits into neither category of aldolase. Cations however, have a profound effect on the activity. With $Tris^+$ (50 mM) and NAD^+ (1 mM) the sole cations (basal conditions) 1 mM K^+ or NH_4^+ stimulated 275 and 400%, resp. Zn^{2+}, Cd^{2+}, and Cu^{2+} (1 mM) abolished activity but the effect of Zn^{2+} and Cd^{2+} was completely reversed by 10 mM EDTA. The effect of Cu^{2+} was irreversible even with 50 mM EDTA. Monovalent cations in the order $Tris^+ < Na^+ < K^+ < Li^+ < NH_4^+$ increased the affinity (decreased the K_m) up to 15-fold for glucose 6-phosphate and 10-fold for NAD^+. The effect of Li^+ on V_{max}, however, was anomalous. Whereas the other cations increased V_{max} in the order $Tris^+ < Na^+ < K^+ < NH_4^+$ up to 250% for glucose 6-phosphate and 440% for NAD^+, Li^+ decreased V_{max} 32% for glucose 6-phosphate with no effect on V_{max} for NAD^+. We conclude that binding of substrate and cofactor are sensitive to the ionic environment.

Significance - Inositol 1-phosphate synthase accounts for all *de novo* synthesis of *myo*inositol, the principal monomeric sugar of the brain. Of the nine possible isomeric inositols, several of which occur in nature, only the *myo* isomer is the precursor of phosphatidylinositol, the phospholipid of membrane uniquely responsive to extracellular stimuli manifested by increased inositol and phosphate turnover. The inositol phospholipids, moreover, are the principal contributors of arachidonic acid, the precursor of prostaglandins. In view of the high level of free inositol (10 mM) normally found in

brain, the anomalous behavior of lithium merits further investigation in explaining the role of the metal in the treatment of mental disorders. The neuropathy associated with diabetes has been attributed to disturbances in the metabolism of either free or bound inositol.

Proposed Course - The reaction catalyzed by the pure enzyme will be studied with the aim of identifying conclusively intermediate ketosugar phosphates. Attempts to crystallize the enzyme, thus far unsuccessful, will continue in order to ascertain by physical methods whether the enzyme is in fact a trimer, as the molecular weights of native and dissociated enzyme suggest. The collaboration underway with Dr. Susan Henry, Department of Genetics, Albert Einstein College of Medicine, New York on identification of products accumulating in synthase-deficient yeast will continue.

Publications:

Eisenberg, F., Jr.: Intermediates in the synthase reaction. In Wells, W. W. and Eisenberg, F., Jr. (Eds.): Cyclitols and Phosphoinositides. New York, Academic Press, 1978, pp. 269-278.

Maeda, T. T., Haegawa, R., and Hayashi, E.: Abnormal lipid metabolism due to *myo*inositol deficiency in rats and yeast. In Wells, W. W. and Eisenberg, F., Jr. (Eds.): Cyclitols and Phosphoinositides. New York, Academic Press, 1978, pp. 451-470.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18002-06 LBM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Investigations on the Biochemical Abnormality Underlying Human Cystinosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dr. Frank Tietze Research Chemist LBM NIAMDD

COOPERATING UNITS (if any)

Dr. Joseph D. Schulman, Dr. E. Jean Butler: Section on Human Biochemical and Developmental Genetics, NPMD, NICHHD.
Dr. Leonard H. Rome, Genetics and Biochemistry Branch, NIAMDD

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SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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1.0	1.0	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Twelve of fourteen strains of cultured skin fibroblasts from patients with I-cell disease (mucolipidosis II) have been found to possess abnormally high intracellular levels of the amino acid cystine and in this respect bear a striking resemblance to the characteristic abnormality encountered in human cystinosis. In contrast to these results nine of eleven human cell lines from patients with the biochemically similar, but clinically less severe, disorder known as mucolipidosis III were essentially normal with respect to levels of this amino acid while two strains exhibited moderately enhanced levels of cystine. Comparative studies of the intracellular localization of excess cystine in cystinotic and I-cell fibroblasts showed that in both cases the amino acid was associated predominately with the lysosomal fraction of these cell types. These observations support the notion that human cystinosis is due to some (as yet unknown) lysosomal dysfunction.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives - To define the biochemical defect which underlies human cystinosis, an often fatal pediatric disorder characterized by excessive accumulation of the amino acid cystine within cells of affected individuals.

Methods - Human cells obtained by skin punch biopsy from patients with cystinosis and other abnormalities, as well as from normal human subjects, are grown continuously in synthetic medium by standard tissue culture techniques. For determination of the intracellular cystine content, washed cells are removed from the culture flasks by treatment with trypsin and disrupted by sonication; the amount of free cystine in the cell lysate is then assayed by a sensitive micromethod which makes use of a specific cystine-binding protein isolated from E. coli. The subcellular location of excess cystine found in cultured cells of patients with cystinosis and I-cell disease (mucolipidosis II) is ascertained after fractionation by one of two methods following preliminary disruption by nitrogen cavitation:
(1) by differential centrifugation of the cell lysates in 0.25 M sucrose according to the classical conditions of De Duve;
(2) by separation of cellular components in a self-generating colloidal silica gradient.

Major Findings - Previous work in this laboratory has indicated that cultured cells from patients with I-cell disease (mucolipidosis II), an abnormality not known to be related to cystinosis, are characterized by excessively high intracellular levels of free cystine. These initial observations have been extended to additional cell lines, with the following overall results: of fourteen I-cell lines examined, twelve have been found with abnormally high cystine content (range: 2-30 nanomoles "half-cystine" per mg protein; normal cells have less than 0.5 nanomoles half-cystine/mg); two I-cell lines were normal with repsect to intracellular cystine. These abnormal levels in I-cells are comparable in magnitude to those seen in cells from cystinotic patients. In contrast to these results, nine of eleven cell lines from patients with mucolipidosis III (pseudo-Hurler polydystrophy), a benign form of I-cell disease, possessed essentially normal levels of cystine, while two of these lines showed marginally elevated levels (1-2 nanomoles half-cystine/mg protein). Cell lines from patients with other lysosomal storage disorders (e.g., Hurler and Hunter diseases; mucolipidosis I) contained normal levels of cystine. Detailed studies of the subcellular distribution of cystine in cultured cystinotic and I-cells indicated that in both types of cells the excess free cystine is associated predominantly with the lysosomal fraction.

Significance to Biomedical Research and the Program of the Institute - Although human cystinosis is classified as a lysosomal storage disease, its biochemical basis remains obscure inasmuch as the metabolism of cystine is not known to involve lysosomal enzymes. The fact that a high proportion

of cell lines derived from patients with I-cell disease, a disorder characterized by multiple acid hydrolase deficiency, also exhibit excessive intra-lysosomal levels of cystine strengthens the concept of cystinosis as a lysosomal storage disorder and to this extent contributes to an understanding of this disease.

Proposed Course - Additional comparative studies will be carried out with cystinotic and I-cell fibroblasts, particularly as they relate to their characteristic property of cystine accumulation. It will be of interest to study the possible "corrective" effects of normal fibroblast secretions on the abnormal cystine levels associated with these mutant cells.

Publications:

Spielberg, S.P., Garrick, M.D., Corash, L.M., Butler, J. DeB., Tietze, F., Rogers, L. and Schulman, J.D.: Biochemical heterogeneity in glutathione synthase deficiency. J. Clin. Invest. 61:1417-1420. 1978.

Tietze, F., and Butler, J.DeB.: Elevated cystine levels in cultured skin fibroblasts from patients with I-cell disease. Pediatric Research, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-18003-06 LBM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Polyamines in the hormone-dependent growth and development of mammary gland.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Takami Oka	Research Chemist	LBM NIAMDD
Other:	Dr. Toshiyuki Takemoto	Visiting Fellow	LBM NIAMDD
	Dr. Yasunori Nagamatsu	Visiting Fellow	LBM NIAMDD
	Mr. John Perry	Research Assistant	LBM NIAMDD
	Mrs. Chiyo Hori	Guest Worker	LBM NIAMDD
	Dr. Masayoshi Ono	Guest Worker	LBM NIAMDD

COOPERATING UNITS (if any)
none

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Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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5.0	4.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Mouse mammary gland contains polyamine-dependent choline kinase, which catalizes the formation of the precursor for the membrane components. With the organ culture system, stimulation of choline kinase activity has been shown to be dependent on the actions of insulin and cortisol, the same combination of hormones that causes accumulation of rough endoplasmic reticulum. The hormonal stimulation of choline kinase activity appears to involve the action of spermidine, a polyamine which accumulates in the cells under the influence of cortisol and mimicks the effect of cortisol on milk-protein synthesis in cultured mouse mammary explants.

2) Lithium ion at 2 to 20mM simulated the stimulatory effects of insulin on cell-proliferation in mouse mammary gland in culture. Lithium ion, however, is incapable of substituting for the action of insulin in augmenting milk protein synthesis. The observed similarities of the response of mammary cells to lithium and insulin suggest the possible importance of cation(s) in the regulation of mammary cell proliferation. On the other hand, the observed inability of lithium to mimic the lactogenic effect of insulin indicates a specific function of the hormone in the functional differentiation of mammary cells.

Project Description:

Objectives - The diamine putrescine and the polyamine spermidine are intracellular polycations synthesized by cells, and are currently considered to play an important regulatory role in the process of growth and development of mammalian cells. Our objective is to assess the functional interrelationship of hormone actions and amines during the development of mammary gland.

Methods - The experimental procedures employed in this investigation include the following: organ culture of mouse mammary gland; preparation of mammary epithelium free of fat cells; isolation and quantification of the polyamines; enzyme assays for ornithine decarboxylase, S-adenosyl-L-methionine decarboxylase, arginase, spermidine synthase and α-lactablumin; a number of column chromatography techniques, such as DEAE, Sephadex, hydroxyapatite, and affinity chromatography; isolation of polysomes and various species of RNAs by sucrose density gradient centrifugation; oligo dT-cellulose and poly U-Sepharose chromatography; characterization of macromolecules by SDS-gel electrophoresis, and development of antibody against casein, α-lactalbumin and S-ado-met decaboxylase and a cell-free polypeptide synthesizing system.

Major Findings - Earlier studies of the action of glucocorticoid in this system have shown that the steroid, in the presence of insulin, facilitates sustained accumulation of rough endoplasmic reticulum (RER), a key site for the formation of milk-proteins. Our present studies show that such action of glucocorticoid involves augmentation of the biosynthesis of phosphatidylcholine, a major phospholipid component of RER of mammary cells. Further, the increased formation of phosphatidylcholine has been shown to be effected through the glucocorticoid stimulation of the activity of choline kinase, which is involved in the formation of the precursors for the membrane components. The hormonal stimulation of choline kinase activity appears to involve the action of spermidine, a polyamine which accumulates in the cells under the influence of cortisol and mimicks the effect of cortisol on milk-protein synthesis in cultured explants.

Previous studies suggested that insulin may exert its effect via changes in plasma membrane structure and intracellular cation concentrations. To gain further insight into the possible role of cations in the hormonal regulation of DNA synthesis in mammary epithelium, we have studied the effect of various inorganic cations on the growth and development of mammary cells in organ culture. Results of these experiments show that lithium ion at concentrations between 2 to 20 mM simulated the stimulatory effects of insulin on the uptake of α-aminoisobutyric acid, synthesis of RNA and DNA, and cell multiplication in mouse mammary gland explants cultured in a chemically defined synthetic medium. Other monovalent cations were virtually ineffective. In most instances, the stimulatory effect of lithium ion is somewhat smaller than and additive to that of insulin. Lithium ion, however, is incapable of substituting for the action of insulin in augmenting milk protein synthesis in mammary explants cultured with other lactogenic hormones, prolactin and glucocorticoid. The observed similarities of the responses of mammary cells to lithium and

insulin suggest the possible importance of cation(s) in the regulation of mammary cell proliferation which may be a common basis for the action of the two agents. On the other hand, the observed inability of lithium to mimic the lactogenic effect of insulin indicates a specific function of the hormone in the functional differentiation of mammary cells.

Proposed Course of Project - An understanding of the action of the polyamines may serve to reveal the complex regulatory mechanisms of tissue growth and development. Such knowledge would certainly be useful for diagnosis and treatment of various pathophysiological disorders, including cancer, and other metabolic diseases.

We intend to pursue further the elucidation of the regulatory mechanism for the biosynthesis of spermidine using antibody for the polyamine biosynthetic enzymes. We are also undertaking a series of experiments to dissect the mechanism of action of polyamines in the cell proliferation and in milk protein synthesis.

Publications:

Oka, T. and Perry, J.W.: Glucocorticoid stimulation of choline kinase activity during the development of mouse mammary gland. Develop. Biol. 68:311-318, 1979.

Oka, T., Sakai, T., Lundgren, D.W., and Perry, J.W.: Polyamines in growth and development of mammary gland. In McGuire, W.L. (ed.): Hormones, Receptors and Breast Cancer, New York, Raven Press, 1978, pp. 301-324.

Hori, C. and Oka, T.: Induction by lithium ion of multiplication of mouse mammary epithelium in culture. Proc. Nat. Acad. Sci. U.S.A. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18004-06 LBM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Biochemical Lesions in Genetic Mucopolysaccharidoses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dr. Irwin G. Leder Research Chemist LBM NIAMDD

COOPERATING UNITS (if any)
Genetics and Biochemistry Branch, NIAMDD,

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A sulfatase which hydrolyzes an O-sulfate ester in a glucosamine derivative bearing a second sulfate on the amino group has been purified from human urine. The enzyme has a pH optimum of 6.3 and is inhibited by chloride and sulfate anions. No activity has been detected in fibroblast extracts.

To establish the specificity of the enzyme and its relationship to physiological substrates, unequivocal synthesis of specifically sulfated α-methyl-2- deoxy-2-sulfamino-6-O-sulfate-D-glucose and α-methyl-2-deoxy-2-sulfamino-4-O-sulfate-D-glucose have been carried out. It has been established that the 6-O-sulfate, the prevalent structure in heparin, is not a substrate for this enzyme.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives - To purify and elucidate the specificity, tissue distribution and role of a glucosamine sulfohydrolase isolated from human urine.

Methods - 1) Standard methods, including molecular sieving, salting out, ion exchange and affinity chromatography are applied to the purification and study of the enzyme.

2) Possible substrates are prepared by sulfation at specific linkages by unequivocal synthetic procedures employing a variety of blocking groups. Compounds labeled with radioactive sulfate are tested as substrates using electrophoresis or ion-exchange procedures to detect and quantitate products of enzymatic hydrolysis.

Major Findings -

1) The disulfated ^{35}S-labeled methyl glucosaminide disulfate prepared by direct sulfation was found to comprise two major components, in a ratio of approximately 7:3, the larger of which was unaffected by the enzyme, whereas the smaller was totally hydrolyzed. To establish the structure of the active substrate, unequivocal synthesis of the N-sulfated α-methyl glucosaminide-6-sulfate and the corresponding -4- and -3- sulfates was undertaken. The -6- and -4- sulfates have been prepared via a series of carbobenzoxy, triphenyl methyl and acetyl blocked crystalline intermediates. The structure of the 6-sulfated derivative has been confirmed by elementary and PMR spectral analysis. Neither of these compounds corresponds to the ^{35}S-labeled substrate prepared by direct sulfation. We may infer that the active substrate is most likely the N-sulfated-α-methylglucosaminide-3-sulfate.

2) A simple column technique has been developed to assay the enzyme replacing the previous procedure based on electrophoresis. The enzyme is maximally active at pH 6.3 and is inhibited by chloride and sulfate ions. No significant activity has been detected in extracts of cultured fibroblasts.

Significance - The sulfatases and other hydrolases, found principally in lysosomes, act sequentially to degrade complex acid mucopolysaccharides. The absence of any one enzyme, may lead to the progressive accumulation of partially degraded polysaccharide fragments and the development of a specific genetic storage disease. The sulfohydrolase described in this study may participate in this process of polysaccharide degradation.

The structural specificity of this sulfohydrolase suggests that similarly sulfated glucosamine residues may be present in mucopolysaccharides or in simpler compounds such as sulfated sugar nucleotides. It has been suggested that canine submaxillary mucin may contain a 3-sulfated glucosamine derivative. Although 3-sulfated degradation products of heparin have not been described, such structures are not excluded since in contrast to dermatan and chondroitin sulfates, the -3- position in the glycosylamine residue of

heparin is available for sulfation. Beyond the fact that the N-sulfate group is essential, but not sufficient for anticoagulant activity, little is known of the relationship of structure to activity in the many roles heparin appears to have in cellular physiology.

Proposed Course: -

1) The N-sulfated-α-methylglucosaminide-3-sulfate will be prepared by sulfation of the 4,6 blocked benzylidine derivative with ^{35}S-labeled sulfate and tested directly for substrate activity.

2) Purification of the enzyme will be advanced and its properties and tissue distribution studied.

Publication: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 18005-06 LBM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Differentiation of Lymphoid Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Milton Kern	Research Chemist	LBM
Other:	Dr. Stephen H. Gregory	Staff Fellow	LBM NIAMDD
	Dr. Sastry Gollapudi	Visiting Fellow	LBM NIAMDD
	Mrs. Blossom Johnson	Laboratory Assistant	LBM NIAMDD

COOPERATING UNITS (if any)
Dr. Daniel H. Zimmerman, Electronucleonics Laboratories, Bethesda, Maryland 20014

LAB/BRANCH
Laboratory of Biochemistry and Metabolism

SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

By combining the technique of immunofluorescence (to label B cells) along with autoradiography (to label [^{125}I]-lipid A binding cells) it has been demonstrated that 20-30 percent of splenic B cells bind lipid A and that other lymphoid cell types in spleen only marginally bind lipid A.

A novel early biochemical event in the activation of thymocytes by concanavalin A has also been demonstrated. Thus freshly isolated thymus cells but not splenocytes secrete a non-immunoglobulin protein in response to concanavalin A.

It has been demonstrated that lipid A induces cells, uniquely present in bone marrow, ta secrete proteins other than immunoglobulins.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives - The purpose of this project is to study the differentiation of the cells involved in the immune response with regard to cell types, cell interactions and control mechanisms. During this report period the experimental work emphasized was as follows: (1) demonstration of the specificity of binding of [^{125}I]-lipid A by certain B lymphocytes, (2) demonstration of a new early biochemical event in the activation of lymphocytes by concanavalin A and (3) demonstration of a lipid A induced secretion of a novel, non-immunoglobulin protein by bone marrow cells but not by other lymphoid sources.

Methods Employed - Secreted products were labeled by incubating cells in medium 199 supplemented with [^{3}H]-leucine and the extracellular fluids were isolated. Secretory products (non-immunoglobulin) were characterized on appropriate Biogel columns. Autoradiographic and immunofluorescence techniques were also employed.

Major Findings - In earlier studies we devised a procedure for iodination of the lipid A moiety of LPS and then utilized the product to establish its preferential binding by B lymphocyte-enriched populations. The present work made use of immunofluorescence (to label B cells) in combination with autoradiography to label cells that bound [^{125}I]-lipid A. It was observed that 20-30 percent of B cells exhibited binding of lipid A, whereas virtually no binding was observed in thymocyte preparations or in θ bearing cells of spleen. B cells are the predominant binding populations in spleen. Indeed, splenocytes other than B lymphocytes ordinarily exhibit marginal binding when detected. The percentage of cells binding lipid A is in accord with the percentage of cells knownto exhibit a mitogenic response. Mouse strains genetically defective in regard to a mitogenic response to lipid A nonetheless bind [^{125}I]-lipid A as well as responder strains indicating that the genetic defect is related to some phenomena other than binding, per se.

Concanavalin A, added to freshly isolated rabbit thymocytes, markedly enhanced the extracellular appearance of non-immunoglobulin proteins. Time course studies revealed that the onset of enhancement occurred virtually without delay. The effect appeared to be restricted only to certain of the thymus-derived cells because thymocytes obtained from rabbits treated with hydrocortisone, as well as splenocytes derived from untreated rabbits essentially did not exhibit the enhancement. Stimulation by concanavalin A was specific in that pokeweed mitogen and lipopolysaccharide were without effect and also in that α-methyl-mannoside, but not galactose, abrogated the concanavalin A-mediated enhancement. Experiments with mouse thymocytes demonstrated that the cells which responded to concanavalin A were primarily cells that bear the θ-antigen on their surface (T-cells).

The lipid A moiety of lipopolysaccharide induced freshly isolated bone marrow cells to exhibit enhanced release of [^{3}H]-proteins other than [^{3}H]-immunoglobulins. Such stimulation by lipopolysaccharide was not observed with cell suspensions from spleen, thymus, appendix, peritoneal exudate

(macrophage), whole blood, reticulocyte-enriched) blood, lymph nodes, liver, testis and the buffy coat of blood. The extracellular appearance of [^{3}H]-proteins was shown to be due to secretion by excluding other alternatives. Thus, the rate of cell death and/or leakage of cellular contents, as well as the rate of shedding of surface membrane protein, was unafffected by lipopolysaccharide. Secretion was selective as judged by the finding that the rate of immunoglobulin release by bone-marrow cells was not enhanced by lipopolysaccharide. The secretory product does not appear to be related to a lymphokine released by macrophage cells and similarily does not appear to have an obligate relationship to the process of mitogenesis. Mouse strain C_3H/HeJ (lipid A non-responder) did not exhibit the enhanced secretion observed by using bone marrow cells of strain C_3H/HeN (lipid A responder).

Significance to Biomedical Research and the Program of the Institute -

A knowledge of the control mechanisms involved in the induction of immunoglobulin production will have vital importance not only in understanding how the organism reacts to foreign antigens, but also may be expected to affect significantly the approach used in regard to a number of immune diseases, e.g., immunodeficiency diseases, allergic disease, autoimmune disease and neoplastic disease of lymphoid cells. An understanding of the activation of lymphoid cells by mitogenic agents like LPS and concanavalin A has direct relevance in characterization of the immune response.

Proposed Course of Project - The product secreted by bone marrow cells in response to LPS will be characterized with respect to its structure and function. The [^{3}H]-proteins will be purified and characterized. Along with these studies, attempts will be made to determine if the secretory product (free of lipid A) has lymphokine activity on bone marrow or spleen cells with regard to a variety of biochemical parameters, e.g., amino acid concentration, DNA, RNA and protein synthesis etc. The possibility that the bone marrow cell secretory product is related to other biological properties of lipid A such as pyrogenicity and adjuvanticity will also be tested. Finally, cell separation techniques will be used to identify the secretory cell in order to determine the possible function of the product i e., is it related to the immune response or not?

Publications:

Kern, M.: Differentiation of lymphoid cells: Evidence for a B-cell specific serum suppressor. Immunol. 35:57-61, 1978.

Gregory, S., and Kern, M.,: Adenosine and adenine nucleotides are mitogenic for mouse thymocytes. Biochem. Biophys. Res. Comm. 83:1111-1116, 1978.

Kern, M., and Gollapudi, S.V.S. : Novel form of T-cell stimulation of immunoglobulin secretion. Nature 276:178-180, 1978.

Gollapudi, S.V.S., and Kern, M.: A novel early effect of concanavalin A on Thymocytes Biochem. Biophys. Res. Comm., in press.

Gregory, S., and Kern, M.: The heterogeneous distribution of 5-nucleotides among rabbit lymphocytes. J. Immunol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 18006-08 LBM

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Enzymes of Carbohydrate Metabolism in Invertebrates and Vertebrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Marjorie R. Stetten	Research Chemist	LBM NIAMDD
Other:	Dr. Paul K. Goldsmith	Research Biologist	LBM NIAMDD

COOPERATING UNITS (if any)

none

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SECTION
Section on Intermediary Metabolism

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The specific activities of four of the key enzymes of rough and smooth microsomes, isolated from endoplasmic reticulular membranes of rat liver and kidney have been measured during the neonatal and early weaning periods of rapid membrane proliferation. The activities of *sn*-glycerol-3 phosphate acyltransferase, NADPH-cytochrome-c reductase and nucleoside diphosphatase remain parallel in the rough and smooth microsomes during postnatal changes in the quantity of enzyme, the rough membranes showing somewhat more activity. This is accord with a picture of enzymes, newly synthesized on ribosome-bearing membranes, being rapidly distributed to the smooth membranes. On the other hand, glucose-6-phosphatase showed non-parallel changes with age in the rough and smooth membranes, the well established neonatal overshoot in activity being almost entirely due to changes in the enzyme in the rough ER. The enzyme activity in the RER reaches a maximum and then decreases after day 2 while that in the smooth membrane is still slowly increasing. It appears that lateral diffusion of glucose-6-phosphatase along the membrane is proceeding at a slower rate than is its replacement by synthesis and degradation within the developing membranes of the neonatal liver.

PHS-6040
(Rev. 10-76)

Project Description:

Studies of some of the enzymes of carbohydrate metabolism in marine invertebrates have been continued. Methods are being developed for the separation of hexokinase isozymes and sugar dehydrogenases of Limulus polyphemus by means of polyacrylamide gel electrophoresis and DEAE-ion exchange chromatography. Since hexose and pentose dehydrogenases appear to be quantitatively more important in the hepatopancreas or midgut glands of those invertebrates which we have studied, e.g. Limulus, starfish, squid, lobster, and clam, than in the livers of vertebrates, we have been trying to evaluate the possible role of these enzymes in the initial stages of the metabolism of sugars in invertebrates. The turnover of muscle glycogen in Limulus has been studied using $U^{14}C$ glucose as a label. Muscle glycogen of Limuli appears to be replaced at a relatively slow rate in well fed animals, the maximum isotope incorporation after a single injection of the labeled compound being reached after about 10 hours and falling to about 50% of the maximum by 48 hours.

Changes in the activities and latencies of four of the key enzymes of the endoplasmic reticular membranes of rat liver and kidney during the neonatal and early weaning periods of rapid membrane proliferation have been studied. The distribution of the phospholipid dependent glucose-6-phosphatase activity between rough and smooth microsomes as a function of age was found to be very different from that of the other enzymes studied. The activities of *sn*-glycerol-3-phosphate acyltransferase, NADPH-cytochrome c reductase and nucleoside diphosphatase remain parallel in the rough and smooth microsomes during postnatal changes in the quantity of enzyme, the rough membranes showing somewhat more activity. This is in accord with a picture of enzymes, newly synthesized on ribosome-bearing membranes, being rapidly distributed to the smooth membranes. On the other hand, glucose-6-phosphatase showed non-parallel changes with age in the rough and smooth membranes, the well established neonatal overshoot in activity being almost entirely due to changes in the enzyme in the rough ER. The enzyme activity in the RER reaches a maximum and then decreases after day 2 while that in the smooth membrane is still slowly increasing. It appears that lateral diffusion of glucose-6-phosphatase along the membrane is proceeding at a slower rate than is its replacement by synthesis and degradation within the developing membranes of the neonatal liver.

The latencies (i.e., fraction of total activity which is not expressed before activation) of two catalytic activities associated with the same enzyme, glucose-6-phosphatase, were found to show strikingly different age related changes in liver but not in kidney. The latency of PP_i-glucose phosphotransferase activity reaches high levels (60-80% latent) soon after birth and remains high throughout life, while the latency of glucose-6-P phosphohydrolase, very high immediately after birth, decreases with age, reaching the adult latency level of about 20% by 10 days. This is a novel observation of different developmental changes in latency for two functions of a single membrane bound enzyme.

Dr. Paul Goldsmith has been engaged in a collaboration with Dr. Anthony Fauci of NIAID in developing enzyme-linked immunoassays for determination of *in vitro* cell mediated specific immune responses. These assays are expected to be useful in determining the functions of lymphocyte subpopulations in patients with immunological disorders.

Proposed Course:

A study of the sugar specificity and kinetic properties of hexose and pentose dehydrogenases, occuring extensively in marine invertebrates, will be carried out, comparing these with properties of the comparable enzymes of vertebrates. Specifically labelled radioactive glucoses will be used to evaluate the relative contributions of the glycolytic and the hexose monophosphate shunt pathways to glucose metabolism *in vivo* in *Limulus*. Isotope incorporation into muscle and hepatopancreas glycogen will be measured in these studies since the concentration of circulating sugars is exceedingly low in marine invertebrates.

Comparative studies will be made of the latency properties of nucleoside diphosphatase, known to be luminally localized in the endoplasmic reticulum, with those of the hydrolytic and phosphotransferase activities of liver glucose-6-phsosphatase. It is hoped that this will permit a decision to be made between the hypotheses that glucose-6-phosphatase is a transmembrane enzyme (Stetten) and that it is a luminal enzyme dependent upon a permease for its activity (Arion)

Publication:

Goldsmith, P.K. and Stetten, M.R: Different developmental changes in latency for two functions of a single membrane bound as a function of age. *Biochim. Biophys. Acta* 583:133-147, 1979.

ANNUAL REPORT OF THE LABORATORY OF CHEMISTRY

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

THE LABORATORY OF CHEMISTRY

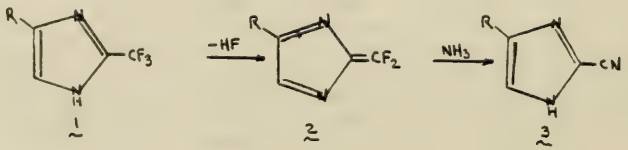
The missions and traditions of the Laboratory of Chemistry, the oldest scientific unit within the NIH complex, have been (1) to engage in research in areas of organic chemistry pertinent to the biomedical and health sciences, (2) to initiate and participate in collaborations with experts in other disciplines, and (3) to provide analytical and consulting services to the NIH community. While all of these activities have undergone significant strengthening and expansion in recent years, there has also been a strong trend toward the development of "in house" skills in biochemistry, molecular biology, and pharmacology. This reorientation has been accomplished by encouraging a number of our organic chemists to acquire experience in the more biomedical disciplines and by including biomedical specialists in the Laboratory staff. The large number of outside collaborations have proved highly successful, but the opportunity of taking a compound from the synthesis stage through microbiological or animal testing, through tissue culture or receptor binding studies, all within the confines of a single laboratory unit, has often proved even more beneficial in bringing a project to fruition. The marked change in the spectrum of activities and goals of our research becomes self-evident upon examination of the project descriptions, summaries, and titles.

In September, 1978, two Sections of the Laboratory were separated and combined into a Laboratory of Bioorganic Chemistry. The remaining five Sections, which make up the "new" Laboratory of Chemistry, are committed to maintain the traditions and eminence of the "old" Laboratory, and to strengthen their efforts to unite research in organic chemistry and in the biomedical sciences into a valuable and quality product.

SECTION ON BIOCHEMICAL MECHANISMS

Chemistry of Imidazoles

2-Trifluoromethylimidazoles (1) are easily obtained by the action of trifluoroacetic anhydride on open-chain precursors, e.g., N,N'-dibenzoylaminoethylenes. Above pH 8, the ring system slowly loses fluoride ion to form a transient difluorodiazafulvene species (2). This highly reactive intermediate



1 2 3

reacts with many nucleophiles to generate new functional groups at C-2 of the imidazole ring. Thus, in aqueous ammonia, the trifluoromethyl group is converted to a cyano group (3) and, in water alone, to a carboxyl group. Loss of fluoride ion from 1 requires strong solvation by five water molecules, resulting in the unique phenomenon that the reactions of 1 are linearly dependent on the water content of the medium, rather than on that of the nucleophile. 4-Trifluoromethylimidazoles show a similar property, losing fluoride ion even faster than the 2-isomers. These properties suggest that trifluoromethyl derivatives of histamine and histidine may serve as irreversible affinity labels for enzyme and receptor sites. To this end, synthetic analogs of LHRF are being prepared containing trifluoromethylhistidines, in the hope that such analogs may combine irreversibly with the LHRF receptor sites. The reactivity of 1 is controlled, in a complex manner, by other imidazole ring substituents and the electronic basis for such control is currently under investigation. [L. A. Cohen, H. Kimoto].

Diazotization of protected 2-aminohistidine and 2-aminohistamine, and treatment of the resulting diazonium salt with azide ion, produces the 2-azido derivatives. Such compounds are of interest, not only as potential competitive inhibitors, but as photoaffinity labels for receptor sites, since the azido group is cleaved to the very reactive nitrene at moderate wave lengths. [R. Jerussi, K. L. Kirk, L. A. Cohen].

N-Triphenylmethylimidazoles react with butyllithium in tetrahydrofuran to generate a carbanion at C-2. Reaction of the carbanion with electrophilic reagents has provided the first syntheses of a variety of new 2-X-histidines and histamines, with X = Cl, Br, I, CHO, COOR, COOH, CH_2OH, and NO_2. These analogs are being screened as potential agonists or competitive inhibitors of the natural imidazoles. Introduction of the nitro group proved to be particularly challenging, since the effective nitrating agents are alkyl nitrates and not nitrites, the normal reagents for such reactions. Equally surprising is the fact that nitration is achieved with the bulky N-triphenylmethyl protecting group, but fails with the much smaller N-methyl group. This method provides the first general synthesis for 2-nitroimidazoles, compounds which have proved to be valuable as antimicrobial and cytotoxic agents, as well as sensitizers for radiation chemotherapy. Synthesis of the histamine and histidine analogs requires elaboration of the side chain of the corresponding 2-X-4-hydroxymethylimidazoles. Current efforts are aimed at the direct introduction of the 2-X-substituent into histidine and histamine themselves. [L. A. Cohen, K. L. Kirk, O. Olubajo, S. Kondo, and D. Davis].

In further studies on the ALP effect in nitrogen heterocycles, kinetic analysis has shown that the decarboxylation of 1-methyl-4-nitroimidazole-5-carboxylic acid follows four distinct pathways, each utilizing a different ionic species. At high pH, decarboxylation of the carboxylate ion proceeds slowly, but no decarboxylation is observed for the isomeric 1-methyl-5-nitroimidazole-4-carboxylic acid. This difference is explained by the fact that the carbanion, which must be generated at C-4, is rendered energetically improbable by electrostatic repulsion of the lone pair at N-3. For analogous reasons, the decarboxylation of 1-methylimidazole-4,5-dicarboxylic acid occurs exclusively at C-5. [H. B. Burkett, K. L. Kirk, L. A. Cohen].

Biochemistry and Pharmacology of 2-Fluoro-L-histidine and Related Compounds

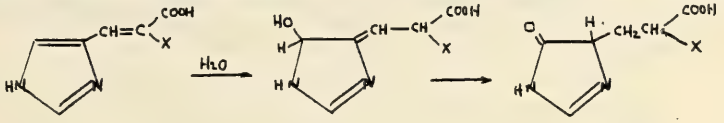
Studies are continuing on the incorporation of 2-F-His into various protein-synthesizing systems, from microorganisms to the whole mouse. Earlier experiments had involved 2-F-His with tritium labeling in the imidazole ring. The stability of the label to exchange is not quite as high as desirable, and efforts are in progress to prepare the same compound with a radioactive label on the amino acid side chain. For this purpose, the corresponding malonic acid derivative has been synthesized; decarboxylation in tritiated water should provide the desired label. [S. Kondo, K. L. Kirk, L. A. Cohen].

In earlier studies, we had found 4-F-His to be a modest substrate for histidine ammonia-lyase, but were somewhat surprised to discover that the deuterium isotope effect for the breaking of the β-CH bond is greater than that for histidine itself. Since the breaking of the β-CH bond must be involved in the rate-determining step for loss of ammonia from the amino acid, the result suggests that, contrary to expectation, fluorine decreases the acidity of the neighboring CH bond. Other investigators have shown that an ortho-fluorine atom reduces the rate of isotope exchange of the methyl group in toluene. Although the latter result is consistent with our enzyme data, it is also conceivable that 4-F-His binds to the enzyme in an anomalous conformation. We have now investigated 4-nitro-L-histidine as a substrate, in which compound the nitro group can be shown to have a considerable acidifying effect on the β-CH bond. Indeed, this compound shows no isotope effect whatever in the enzymatic abstraction of β-hydrogen, supporting our assumption that the ease of abstraction of the β-hydrogen is governed largely by electronic effects. Furthermore, the rate of formation of 4-nitrourocanic acid is no longer dependent on the concentration of added metal ion, implying that the acidity of the β-hydrogen is now so great that its abstraction now precedes the rate-limiting loss of ammonia. [C. B. Klee (NCI), K. L. Kirk, L. A. Cohen].

We had previously suggested that the antileukemic effect of 2-F-His may be related to the powerful inhibitory effect of its metabolite, 2-F-urocanic acid, on urocanase. The important consequence of such inhibition would be a temporary interruption in the utilization of histidine as a one-carbon source for purine biosynthesis. Further examination of this postulate required the availability of sizable quantities of 2-F-urocanic acid, much more than could be prepared enzymatically. A chemical synthesis has now been developed, involving aryl-diazonium coupling at C-2 of urocanic acid, stannous chloride reduction of the azo compound to 2-aminourocanic acid, and photochemical conversion of the latter compound to 2-fluorourocanic acid. Conjugates of this compound, which may be able to penetrate cell membranes, are being explored. [O. Olubajo, K. L. Kirk, L. A. Cohen].

During the enzymatic conversion of urocanic acid (4, X = H) to imidazolone-propionic acid (6), the vinyl proton α to the carboxyl group is converted transiently to an allylic proton (5, X = H). Since allylic halides, in contrast to vinyl halides, are very susceptible to nucleophilic attack, it seemed reasonable to prepare 4 (X = halogen) as potential suicide substrates for urocanase. This compound has now been prepared with X = Fluorine via a Wittig condensation. The

analog is neither a substrate nor an inhibitor, and failed to retard the production of urocanic acid by histidine ammonia-lyase. Since the high electronegativity of fluorine may preclude protonation of the double bond (possibly the first step in the enzymatic protonation), studies are in progress with the less electronegative chloro and bromo analogs. [E. Pearcy, K. L. Kirk, L. A. Cohen].



4 5 6

By use of our photochemical method for the introduction of aromatic fluorine, a convenient and direct route to optically active analogs of aromatic amino acids has been devised. In this way, 3-fluoro-L-tyrosine, 3,5-difluoro-L-tyrosine, and 5-fluoro-L-DOPA have now been prepared [K. L, Kirk].

Chemistry and Biochemistry of Fluorinated Biogenic Amines

The three ring-fluorinated analogs of dopamine (FDA) have been synthesized and subjected to biological evaluation: 2-FDA, 5-FDA and dopamine (DA) are equipotent as DA vascular receptor agonists, on the basis of the increase in renal blood flow of phenoxybenzamine-treated dogs, while 6-FDA is ca. 4-fold less potent. The three analogs are comparable to DA in α-adrenergic agonist potency, as measured (in vivo) by femoral blood flow in the dog; similar results are obtained for β1-adrenergic agonist activity, as measured by in vivo cardiac contractural response. Response in the central nervous system (CNS) was determined by comparing the abilities of 2-, 5-, and 6-FDA, relative to DA, to displace specific radioligands in brain slices. Again, 2-FDA, 5-FDA, and DA were equipotent, with 6-FDA showing some diminished activity. Thus, it would appear that the presence of fluorine at the 2- or 5-position of the catechol ring has no significant effect on biological activity, while the 6-fluoro analog is somewhat weaker in two of the three tests.

The dramatic differences in agonist specificities previously observed for the isomeric ring-fluorinated norepinephrines (FNE) have been investigated in greater detail. Peripheral studies (in vitro) have confirmed that 5-FNE, 6-FNE, and NE are equipotent as α-adrenergic agonists (guinea pig aorta), while 2-FNE has only marginal activity. 2-FNE and NE are equipotent as β-adrenergic agonists, 5-FNE is ca. seven times more potent, while 6-FNE is essentially inactive. In vivo experiments agreed with the in vitro results. Thus, the effects of 2-, 5-, and 6-FNE on cardiac contractural force (β1-adrenergic response), arterial blood pressure (β2-adrenergic response), and femoral blood flow (α-adrenergic response) were compared to those of NE in anesthetized dogs. The results demonstrated that 2-FNE acts on β1 and β2 adrenergic receptors, 5-FNE on α and β1 receptors, and 6-FNE only on α-receptors.

Extension of these studies to the CNS was accomplished by examining the fluoro analogs in the cyclic-AMP generating system in rat brain slices. The results show that the 2-FNE response corresponds to that of the β-component

(propranolol-sensitive) of the system, while 6-FNE gives a response corresponding to that of the α-component (phentolamine-sensitive). 5-FNE behaves similarly to NE (α- and β-responses). The effects of FNE's on binding of radioactive α- and β-adrenergic antagonists to rat cerebral cortical membranes confirm the selectivities of their action in the CNS, and suggest that the specificities may result from binding selectivity. [K. L. Kirk, D. Cantacuzene, C. R. Creveling, J. Daly, D. McColloh, Y. Nimitkitpaisan, W. Padgett, E. McNeal, L. I. Goldberg (U. Chicago) and J. D. Kohli (U. Chicago)].

The value of the ring-fluorinated biogenic amines as pharmacological tools has been amply demonstrated. In addition, correct interpretation of the causes of the specificities observed may give insight into the nature of specific receptor-agonist interactions and/or receptor structures. Physical chemical studies on the isomeric FNE's, designed to determine the molecular effects of fluorine substitution, are in progress. Analogs with additional structural modifications are being prepared to examine the relationships of fluorine substitution to other structural parameters which have already been identified. Thus, α-methyl-FNE's and N-isopropyl-FNE's (fluoroisoproteronol) have been synthesized for adrenergic agonist specificity studies. The corresponding ring-fluorinated DOPA's are being prepared for in vivo CNS experiments. [K. L. Kirk, and D. Cantacuzene].

Melatonin (N-acetyl-5-methoxytryptamine) is a potent inhibitor of luteinizing hormone-releasing factor (LHRF). Hydroxylation of melatonin at the 6-position destroys its activity toward LHRF. 6-Fluoro- and 4,6-difluoromelatonin have been synthesized and investigated in pituitary glands with the hope that the stronger C-F bond would delay inactivation, resulting in a longer lasting drug. Initial results indicate that 6-fluoromelatonin and melatonin are equipotent in inhibition of LHRF, while 4,6-difluoromelatonin is 0.03 times as active. In vivo studies show that all three compounds have virtually identical biological half-lives. [K. L. Kirk, J. E. Martin (Washington U.) and D. C. Klein (NICHD)].

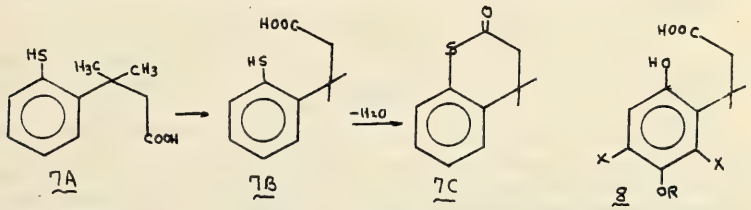
F^{19} NMR Spectra as Probes of Molecular Motion

F^{19} NMR studies of fluorinated molecules in intact cells have continued. Within blood platelets are found membrane-enclosed granules which contain high concentrations of 5-hydroxytryptamine (5-HT), along with adenine nucleotides and divalent metal ions. F^{19} NMR spectra of fluorinated-5-HT, incorporated into the granules of both human and pig platelets, have shown that the motional state of the 5-HT is restricted. On the basis of P^{31} NMR data (adenine nucleotides), it would appear that high molecular weight aggregates are formed between the nucleotides and metal ions, and that these aggregates further bind 5-HT, restricting its motion. [K. L. Kirk, J. L. Costa (NIMH), C. M. Dobson Harvard U.), F. M. Poulsen (Harvard U.), C. R. Valerie and J. J. Vechione (Naval Blood Research Lab., Boston)].

Stereopopulation Control and Models for Enzyme Action

The kinetics of thiolactonization of 3-o-mercaptophenyl-3,3-dimethyl propionic acid gives nonlinear rate plots, the degree of curvature increasing

with the time of aging of the alkaline stock solutions. This puzzling phenomenon has been clarified by the observation that the nmr spectrum of the alkaline solution also undergoes a time-dependent change. Electrostatic repulsion between the negative charges in the dianion (S^-, COO^-) drives the normally cisoid conformation of the propionic acid side chain into an extended transoid conformation. After acidification and neutralization of the negative charges, the side chain (7A) slowly reverts to the thermodynamically preferred (and kinetically productive) cisoid conformation (7B) with $t_{1/2}$ = 22 min. Thus, the complex kinetics for thiolactonization must result from the combination of two



7A 7B 7C 8

separate processes: the rapid cyclization of that fraction of substrate which already exists in form 7B and the slow conformational isomerization of 7A to 7B (eq 1). Computer analysis of 70 kinetic runs has permitted the evaluation

$$\text{rate} = A \xrightarrow{k_1} B \xrightarrow{k_2} C \quad + \quad B \xrightarrow{k_2} C \qquad (1)$$

of the rate constants, k_1 and k_2, as well as the determination of the cis/trans ratio in each stock solution. The rate constant for the cis $\longrightarrow$ trans conversion (by nmr) is identical to that for the trans $\longrightarrow$ cis conversion (by kinetic analysis of ring closure). The barrier to facile rotation (ca. 13 kcal per mole) must arise from the bulk of the sulfur atom, since the analogous phenolic system fails to show a slow conformational change. This is the first example ever reported of an intramolecular reaction with a conformational change as the rate-limiting step. A search for other cases is in progress. The phenomenon has considerable implications in protein chemistry and enzymology, since it serves to demonstrate by simulation that conformational change in a protein, or induced fit by a substrate, can sometimes be slow enough to serve as the rate-limiting step in enzymatic reactions. [M. Blum, M, Channing, L. A. Cohen].

We have attributed the extremely high rates of lactonization found for certain phenolic acids (series 8) to rigid cisoid geometries; thus, they may be considered to simulate the enzyme-substrate complex. Other investigators have argued that these very fast ring closures are due, primarily, to relief of overcrowding by ring formation. As part of an effort to generate support for our interpretation, we have now shown that the rates of ring closure are practically the same for X = Cl, Br, or CH_3 in series 8. Since these substituents vary significantly in size, it seems rather unlikely that all the compounds would show the same compulsion to relieve steric crowding. Furthermore, chlorination of the phenolic system (R = X = H) produces isomeric monochloro derivatives in equal amount; hence, the steric environment on either side of the phenol cannot be radically different. Bromination first produces the uncrowded monobromo

derivative at low temperature; further bromination gives the dibromo derivative with the rate constant for the second bromination being even faster than that of the first. Hence, the bromine atom is not rejected because of overcrowding. Finally, nitration of 8 (R = CH_2Ph, X = H) produces isomeric mononitro derivatives and thus, even the nitro group does not encounter a crowded environment. [M. M. King, L. A. Cohen].

SECTION ON CARBOHYDRATES

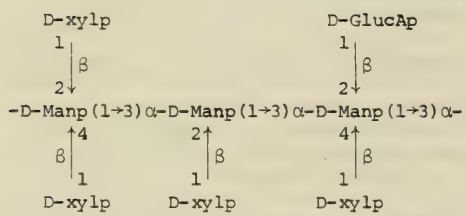
The Nature of Binding of Ligands to Immunoglobulins.

It has been shown that neutral carbohydrate ligands probably bind to immunoglobulins by hydrophobic bonding, mediated by the mutual exclusion of solvent. O-glycosides of galactose having an aromatic aglycon appear capable of interacting with the aromatic amino acid residues located in the combining area.

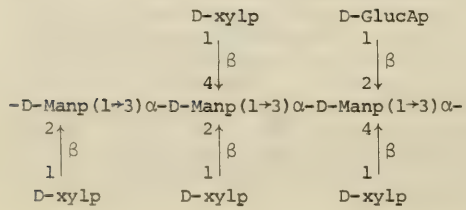
Charged ligands such as phosphorylcholine bind to their immunoglobulins through charged interactions. These are greatly influenced by the hydrophobicity of groups located near the charge on the ligand. It is suggested that these groups act by displacing water from the combining pocket thereby lowering the local dielectric and hence increasing charged interactions. It was also shown that the size of the binding site of antiphosphorylcholine antibody appears much more flexible than was originally believed (M.K. Das, E. Zissis and C.P.J. Glaudemans).

Structural Studies on Fungal Antigens.

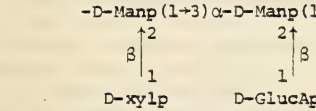
The structure of Cryptococcus neoformans type C was refined to be either:



or:



The structure of the antigen from Cryptococcus neoformans type D can be represented as:



(A.K. Bhattacharjee, K.J. Kwon-chung and C.P.J. Glaudemans).

Interaction of Dextran with Immunoglobulin W3129

This immunoglobulin is unusual in that it only binds to the non-reducing terminal end of a linear polysaccharide antigen (dextran α1,6). This was suspected by earlier workers (Kabat et al.) who found that linear dextran did not precipitate with the antibody. We have confirmed this by the fact that isomaltopentaose and the polymeric dextran show identical constants of association with the antibody W3129 when calculated on the basis of their respective molecular weights (L.G. Bennett and C.P.J. Glaudemans).

SECTION ON MEDICINAL CHEMISTRY

Our Section continues to be strongly involved in the areas of natural opioids, natural and synthetic antitumor agents, false transmitters which interfere with dopamine, and irreversible binding agents to the benzodiazepine receptors. Some of the problems encountered are of a purely chemical nature, whereas others are related to the question of how these molecules interact with biopolymers on a molecular level, or how they are being biotransformed and metabolised and require collaboration with biochemists, pharmacologists and biologists. Progress in these different areas is discussed below. The biological testing program for analgesic activity, toxicity, dependence liability and narcotic antagonism is continuing. Analgesic activities are measured by the hot-plate and Nilsen methods. The more active compounds are further assessed for physical dependence and antagonist properties in Rhesus monkeys and in vitro binding to the opiate receptor. More than one hundred compounds from universities, the Laboratory of Chemistry and the world-wide pharmaceutical industry have been submitted to this program this year. About 50 compounds were assessed for physical dependence liability and narcotic antagonist properties. (L. Atwell, A. E. Jacobson, Committee on Problems of Drug Dependence, Inc.)

Our Section is involved with research which could lead to a technical synthesis of natural morphine and related congeners, especially codeine. It is aimed at producing the natural alkaloid from readily available chemicals. Although we realize that this goal is pursued by several other groups, we feel that our experience gives us a good chance to make a valid contribution toward this objective.

Total Synthesis of Morphine

Since our research has made pure N-norrecticulines available, the biosynthetic approach to morphine was reinvestigated. The aim was to improve the yield in phenolic oxidative coupling from N-norreticuline derivatives to salutaridine derivatives. Although we could not accomplish that, some interesting discoveries were made which provided an entry into the classes of isopavinan and pavinan alkaloids (discussed below). The approach to morphine from N-norreticuline has not been abandoned, but is being pursued with an outside collaborator. It was found that the formation of aporphines in the phenol oxidative cyclization can be avoided and the yield of salutaridine from N-norreticuline could thus be pushed to more than 30%. We have concentrated on a modified Grewe approach, already executed in principle by Professor Beyerman in Holland. We were stimulated to reinvestigate this scheme by our study of 3-deoxydihydromorphine, a compound reported earlier. This morphine congener was converted by oxidation into its ketone which was transformed to (-)-4-hydroxy-6-ketomorphinan. Since the latter could be reconverted into 3-deoxydihydromorphine by bromination followed by catalytic reduction, a total synthesis of morphine along these lines will now concentrate on the following two issues: A practical conversion of 3-deoxydihydromorphine into dihydromorphine and a practical synthesis of (-)-4- hydroxy-6-ketomorphinan. Both aspects are being pursued. (A. Brossi, F. Hsu, A. E. Jacobson, M. Reich and K. C. Rice).

Metabolism of 3,6-Dideoxydihydromorphine

The question of whether the observed strong antinociceptive activity of this compound in mice involves a metabolic introduction of the phenolic group in the 3-position is presently being discussed. A metabolic study in monkeys, planned with collaborators from the University of Mississippi should show whether the metabolites belong to the 3-deoxy series or to the dihydromorphine series. The urinary extracts of animals treated with 3,6-dedeoxydihydromorphine will be analyzed and the major metabolites isolated and identified. To be continued. (A. Brossi, A. E. Jacobson, M. Rozwadowska, K. C. Rice).

Biomimetic Type Synthesis of Isopavinans and Pavinans

These are rare isoquinoline alkaloids; their pharmacology has not been fully investigated. The racemic and optically active N-norreticuline derivatives (N-carbethoxy and N-formyl), utilized in the total synthesis of morphine, are being methoxylated in the benzylic position of the tetrahydroisoquinoline (TIQ) *via* oxidation with DDQ (dichlorodicyanoquinone). The methoxylated TIQ were isolated in the N-formyl series as crystalline compounds. Acid catalysis afforded the isopavinan system, and the intermediates were easily converted into racemic and natural (-)-thalidine. The latter was identical with the natural alkaloid. Thermolytic elimination of the methanol from the 4-methoxylated TIQ provides an entry into the pavinan series. Thus, the 1,2-dihydroisoquinolines obtained from the 4-methoxylated N-norreticulines afforded the intermediate N-acyl-pavinans by heating, and racemic and natural (-)-bisnorargemonine after acid catalyzed cyclization and reduction with LAH or diborane. Both classes of alkaloids can be obtained regioselectively by this procedure and were obtained in high yield and, in the case of the optically active isomers, optically pure. Since hydrazinolysis of the intermediate N-acyl-N-noralkaloids affords the secondary amines, structural variation in these

molecules is possible. Some of these compounds are being evaluated pharmacologically. Project will be continued if interesting activities are found. (A. Brossi, A. E. Jacobson, J. Reden, K. C. Rice, W. Ripka).

Selective Ether Cleavage of Polyoxygenated Aporphines

It has been found in model compounds lacking a basic nitrogen function that an aromatic methoxy group next to a methylenedioxy function can be cleaved with trimethylsilyl iodide in quinoline. In systems containing basic nitrogen functions quinoline has to be replaced with a high boiling solvent and a strong base, such as DABCO, in order to avoid the formation of alkaloidal quaternary by-products. The well-known aporphine alkaloid S-(+)-laureline could thus be O-demethylated into S-(+)-mecambroline, representing a partial synthesis of this alkaloid. Project completed. (A. Brossi and J. Minamikawa).

By-product of the PPA Catalyzed Cyclization of Dihydrosinomenine

The structure of a by-product obtained in the acid catalyzed cyclization of (+)-dihydrosinomenine and obtained in about 10% yield was elucidated. It could be shown that the crystalline material was an unsaturated 7-ketomorphinan which existed in solution in equilibrium with its oxygen bridged tautomer. The unusual behavior of this material in solution was followed by physical studies, and the postulated structure of the crystalline material confirmed by X-ray analysis. Project completed (A. Brossi, A. E. Jacobson, I. Iijima, J. Minamikawa, K. C. Rice, J. Silverton and T. Williams).

Mammalian TIQ Alkaloids

Tetrahydropapaveroline (THP) is the most interesting of the "Mammalian Alkaloids" detected in the urine of patients with Parkinson's disease. Its synthesis in racemic and optically active form from N-norreticulines has been achieved and material is easily available. THP acts as a false transmitter of dopamine. THP is unstable in aqueous solution above pH 7.2 and undergoes spontaneous decomposition in the presence of oxygen. Among the decomposition products, dibenzopyrrocolines were detected and isolated as their polyacetates. The tetraacetate obtained is identical with known material. The second product, isolated and identified, is a dimer; its structure was established by careful physical investigation. In order to discern whether THP is formed in a Pictet-Spengler condensation between dopamine and dopa aldehyde, as is generally assumed, or by the biosynthetic pathway prefered for the synthesis of benzylisoquinolines by plants, which involves TIQ-carboxylic acids as intermediates, two monomethylated THP's were synthesized. The 6-O-methylether of THP obtained should be a minor metabolite of THP if the latter is formed in a direct condensation, whereas the 3'-O-methylether should be present if THP is formed by the carboxylic acid pathway. The 1-carboxylic acid of THP is a major metabolite in man. Both O-methylethers are valuable compounds for diagnostic purposes. Catecholic pavinans and isopavinans were prepared from intermediates of our synthesis and could in principle originate from THP by benzylic oxidation followed by cyclization. To be continued. (A. Brossi, A. E. Jacobson, P. Mak, J. Reden and K. C. Rice).

Natural and Synthetic Antitumor Agents

Colchicine:

Our studies have shown that unnatural (+)-colchicine does not bind as well to rat brain microtubule protein as its natural enantiomer. An easy conversion of colchicine into demecolcine was accomplished, and colchicine derivatives with amino- and substituted amino-functions in the 7-position are now readily available. The many compounds prepared were evaluated by binding to rat brain microtubule protein, using colchicine as a standard. Those with binding similar to colchicine were examined for their in vivo toxicity in mice, after i.p. injection. From a series of about 30 derivatives, about six showed relatively good binding and were considerably less toxic than colchicine. These compounds are now being evaluated in vivo at NCI for antitumor activity. In connection with these studies, several rare colchicum alkaloids were prepared in quantity, including demecolcine, methyl-demecolcine and speciosine.

The finding that deacetamidocolchicine, lacking the acetamido side chain, binds well in vitro is interesting, and prompted the synthesis of several dehydro analogs. This groups will be extensively studied since they all showed good in vitro binding and were less toxic than colchicine. From the three known phenolic congeners of colchicine, only the 2-O-demethylcolchicine showed good tubulin binding affinity, whereas the 1-O-demethyl and the 3-O-demethyl analogs were substantially less active. An efficient synthesis of the 2-O-methyl-colchicine is now feasible, and this compound and derivatives will be further investigated.

Based on presently available knowledge it seems likely that a much less toxic colchicine can be prepared. These data have, however, to be substantiated. This project will be continued. (A. Brossi, H. Capraro, A. E. Jacobson and M. Iorio).

Retinoids:

Retinylidene-dimedone has emerged as a standard in our series of condensation products of retinal with 1,3-diketones. Enlargement of the six membered terminal group with a 7-,8- and 9-membered ring 1,3-diketone system has shown that activity is sustained and that these compounds, contrary to the finding with retinylidene-dimedone, afford much stronger tissue coloring. The synthesis of even larger rings, which often necessitates the development of new chemical syntheses since the required 1,3-diketones are rarely known, is being continued. Modification of ring A in retinylidene-dimedone has given the expected epoxide and its dihydrofuran rearrangement product. Both seem less active than the parent compound. None of the many derivatives of retinoic acid with different terminal end groups (amides, esters etc. prepared) have shown increased activity.

The search for more active, well tolerated and relatively stable derivatives of retinal, with potential use as prophylactic antitumor agents, is being continued. (A. Brossi, N. Acton, A. E. Jacobson).

Inhibitiors of Benzodiazepine Receptor Sites

Benzodiazepines, represented by Librium and Valium, have been shown to interact reversibly with high affinity binding sites in the mammalian CNS. Their anticonvulsant and anxiolytic activity seems to be related to this interaction. In order to further characterize these receptor sites, benzodiazepines were synthesized which act specifically and irreversibly with them through covalent bond formation. Receptors labeled by such interaction can be differentiated from other proteins, and their characterization will be of value in isolating and characterizing these receptors. Irazepine, the first representative of this class, contains a reactive isothiocyanate function. It reacts irreversibly with benzodiazepine receptor sites in vitro. A second generation of reactive benzodiazepines suggests that there exist more than one benzodiazepine receptor, which may explain the two different principle pharmacological effects observed with benzodiazepines (anticonvulsant and anxiolytic activity). This project is being continued. (K. C. Rice, A. Brossi, P. Skolnick).

SECTION ON METABOLITES

The section's major thrust is concerned with the mechanism of induction and action of interferon and interferon-induced, double-stranded RNA-activated enzymes (Torrence, Johnston, Imai in collaboration with Epstein and Friedman of LEP/NIAMDD and Zoon from LCB/NIAMDD). Two such enzymes of interferon-treated cells are the 67K protein kinase and the 2'5'-oligoadenylate synthetase. These activities were first described in the laboratories of Lengyl (Yale), Revel (Weizmann) and Kerr (Mill Hill). They have attracted considerable attention for the role they may play in the action of interferon and the dsRNA-mediated inhibition of protein synthesis seen in extracts of interferon-treated cells. Torrence and Friedman recently completed a study of the dependence of the latter phenomenon on nucleic acid structure. With these data in hand, we are currently attempting to determine which of the polymers that are potent inhibitors of cell-free protein synthesis can activate the 67K protein kinase and which can activate the 2',5'-oligoadenylate synthetase. The relative importance of these two systems could thus be ascertained. One finding of substantial interest is that, under certain conditions, $(I)_n.(br^5C)_n$, completely inactive as an inhibitor of protein synthesis, is just as potent an activator of the kinase as is $(I)_n.(C)_n$, a potent inhibitor of protein synthesis. However, under protein synthesis conditions, $(I)_n.(C)_n$ is by far more effective as a kinase activator. This apparent anomaly has been resolved by the finding that a phosphoprotein phosphatase is active under protein synthesis conditions and, in the case of $(I)_n.(br^5C)_n$, this phosphatase is much more active than in the case of $(I)_n.(C)_n$. Thus the rate of dephosphorylation as well as the rate of phosphorylation is of paramount importance in determining net ^{32}P incorporation into 67K protein.

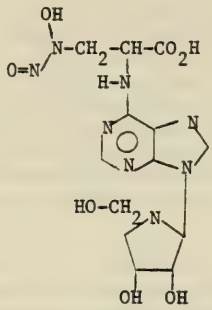
Other studies in the Metabolites Section are more chemical in nature.

The Synthesis of Indolizidine Alkaloids

The Lions-Williamson synthesis is being applied to the preparation of 3-n-butyl-5-n-propylindolizidine, an alkaloid found in skins of the frog

Dendrobates histrionicus (J. Daly). A new synthesis of a key intermediate for that synthesis, 3-ketooctanal dialkyl acetals has been devised. Such compounds should prove useful in the synthesis of 2-alkyl furans (Spande).

The alanosine-derived nucleoside (A) is being synthesized in a scale suitable for biological testing (P. Torrence, J. Imai). Modifications of a synthesis previously developed (T. Spande) will be studied.



(A)

Synthetic Photochemistry and Photobiology

The synthesis of novel heterocycles of metabolic significance and with potential for antiviral and CNS activity has been accomplished by photocyclizations and dimerizations (Witkop)

Novel tryptophan metabolites were synthesized and their relative and absolute stereochemistry and chirality established by Roentgen Ray Analysis (Witkop).

Central Nervous System Agents Based on the Piperidine and Pyrrole Ring Systems

Aromatic and heterocyclic esters of the non-quaternary carbon-4 piperidinol system exhibited analgestic activity in mice, no physical dependence liability of the morphine-type in monkeys and rats and little or no binding to the opiate receptor. A 3-substituted piperidine of this series showed narcotic antagonist properties similar to that displayed by Cyclazocine. Piperidinol esters, dissimilar to those displaying good analgesic activity, were active in the maximal electroshock antiepileptic assay in mice. Pyrrole-carboxylic acids showed good activity in anti-inflammatory and antiproteolytic assays. Certain substrates within these three disciplines are being studied for their respective receptor interactions (Waters).

SECTION ON MICROANALYTICAL SERVICES AND INSTRUMENTATION

Service Function and Instrumentation

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are determined on a quantitative basis, using ultramicro, micro, and inorganic research samples, commercial perparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents when requested. Instrumental analyses include: GC/MS spectromometry, gas-liquid chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, atomic absorption spectrometry, amino acid analysis, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colorimetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request (D.F. Johnson, H.J.C. Yeh, W.R. Landis, P. Parisius, B. Baer, A. Wong, N. Whittaker, and B. Torain).

Characterization of the Dehydrogenase-Isomerase Enzymes and Metabolic Pathways in Tetrahymena Pyriformis

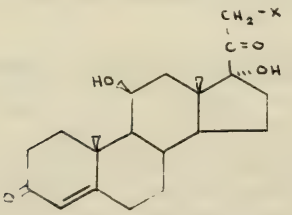
The protozoan Tetrahymena pyriformis has steroid dehydrogenases that are similar in functional properties to the dehydrogenases of higher eucaryotes. Specifically, our prior identification of 4-androstene-3β,17β-diol and pregnenolone as transformation products from the incubation of T. pyriformis with testosterone and progesterone, respectively, indicated that this protozoan possesses a dehydrogenase-isomerase system that can isomerize a Δ^4-steroid to a Δ^5-steroid and reduce the C-3 carbonyl to a 3β-hydroxyl. The characterization of this system is being pursued in several ways.

The β-hydroxysteroid dehydrogenase (β-HSDH) is obtained as a crude supernatant from a 100,000 x g centrifugation of ruptured cells. Purification of the supernatant involves chromatography on a DEAE cellulose column, ammonium sulfate precipitation, and affinity chromatography on blue sepharose. Although the amount of this enzyme activity in T. pyriformis is small, the development of a quick spectrophotometric assay and availability of large fermentors is making the purification feasible. Once a sufficient amount of β-HSDH activity is partially purified it will be used for kinetic and substrate specificity studies.

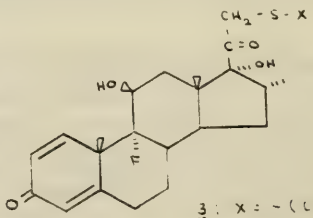
Preliminary data indicate that the partially purified β-HSDH is specific for the 3β-hydroxyl of a steroid, but the presence of that functional group is not sufficient for binding. Although chlosterol was 100 to 1,000 times more reactive than 4-androstene-3β-17β-diol with the partially purified β-HSDH, there was no response to cholesterol with a more purified preparation. Thus it is possible that T. pyriformis possesses more than one 3β-HSDH. Further substrate specificity studies will be done to determine the structural requirements for activity of a 3β-hydroxysteroid with a purified preparation of β-HSDH (N.S. Lamontagne and D.F. Johnson).

Nature of Steroid-Receptor Interactions

Steroid specific events are induced by nanomolar concentrations of hormone in responsive cells. Thus, extremely sensitive methods are required in order to monitor the steroid at physiological levels. Fluorescence spectroscopy can 1) detect some compounds at concentrations as low as 10^{-12} M and 2) provide information about the microenvironment of the fluorescent molecule. These two properties make fluorescence spectroscopy ideally suited for a study of steroid-receptor interactions. Due to the tendancy of receptor-steroid complexes to dissociate, a covalently bound fluorescent receptor-steroid complex would be even more useful. In order to obtain such a complex, we have developed a new methodology for affinity labelling since the addition of a specific chemical is required to initiate the affinity labelling process. Chemo-affinity labelling (CAL) thus combines the ability to control the timing of covalent bond formation, a distinguishing feature of photo-affinity labelling (PAL) with the advantages of the relatively stable and specific "reactive" functional groups found in conventional, or electrophilic, affinity labelling (EAL). Our chemical initiator for the chemo-affinity labelling process is o-phthalaldehyde (OPTA), which should also be useful as a new bi-functional, chemical cross-linking reagent. OPTA rapidly reacts with primary amines and thiols to give 1-alkylthio-2-alkylisoindoles in high yield (Simons, S.S., Jr. and Johnson, D.F. (1978) J. Org. Chem., 43, 2886-2891). [In collaboration with Ruth Doherty of Univ. of Md., we are trying to obtain an x-ray structure of an isoindole derivative in order to unambiguously prove the iosindole structure and to answer some basic theoretical questions about this ring system.] Since these isoindoles are usually intensely fluorescent, (Simons, S.S., Jr. and Johnson, D.F. (1978) Anal. Biochem., 90, 705-725), this chemo-affinity labelling reaction has been named fluorescent chemo-affinity (FCA) labelling, or FCAL. In order to obtain a fluorescent, covalent receptor-steroid complex, we have prepared three hydrolytically stable glucocorticoid derivatives (1-3) which should participate in the FCAL reaction. In model



2: X = SH



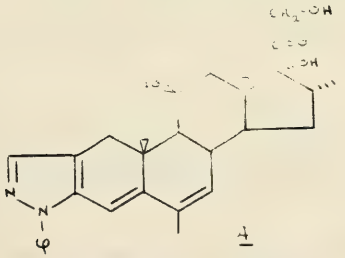
3: X = $-(CH_2)_4-SH$

4: X = $-(CH_2)_3-NH_2$

studies with OPTA and an added amine or thiol, each steroid gives a good yield of the desired isoindole, two of which (derived from 2 and 3) are stongly fluorescent and display good stability and response to changes in solvent polarity. In collaboration with Dr. Thompson, each steroid displayed high affinity for the glucocorticoid receptors from rat liver hepatoma tissue culture (HTC) cells and induced the enzyme tyrosine aminotransferase (TAT). Thus these synthetic steroids show excellent promise for being effective FCA labels for glucocorticoid receptors.

We have previously reported studies (in collaboration with Dr. Thompson) on two novel cortisol derivatives (cortisol-21-thiol acetate and -21-(O-ethyl) xanthate) which potentially could transfer a small functional group (i.e., acetyl or thionocarbethoxy) to the glucocorticoid receptor to give a functionally active, covalently labelled receptor. Since we have been unable to obtain the necessary radio-labelled cortisol derivatives, we have further examined these steroids by comparing their properties to that of the expected cleavage product, cortisol-21-thiol. The relative affinities of these steroids for receptors did not correlate with the bulk of the steroid substituents since the thiol, thiol acetate and xanthate derivatives were all approximately equal in being better competitors of ^{3}H-dexamethasone binding than the parent steroid cortisol. Interaction of the latter two steroids with HTC cell receptors was further examined in a cell-free exchange assay that renders steroid-free receptors incapable of binding the subsequently added ^{3}H-steroid. The behavior of the chemically reactive thiol acetate and xanthate derivatives in this exchange assay, their unexpectedly high apparent affinity for receptors, and the similarity of their cell-free and whole cell properties with those of cortisol-21-thiol are all consistent with the reaction of cortisol-thiol acetate (or -xanthate) with the receptor to yield a functionally active, covalently labelled glucocorticoid receptor and cortisol-thiol.

In an effort to discover steroids with even higher affinities for receptor that could be used in the above studies, we examined the pyrazolo-steroid 4, which is an extremely potent anti-antiflammatory steroid but was predicted to be an inactive glucocorticoid on the basis of structure-activity data. We



4

have now shown that 4 is also a potent glucocorticoid in a well defined system. The observed high affinity of 4 for the HTC cell receptors indicates that the current concepts of glucocorticoid structure-activity should be revised. The close correlation of biological activity of 4 in anti-inflammatory and TAT induction assays suggests that the latter assay using the HTC cells, might be a much more convenient and rapid screening test for anti-inflammatory steroids than the presently used, more cumbersome assays. Furthermore, the close correlation between the two assays supports the hypothesis that both glucocorticoid induced responses are mediated by specific steroid receptors (S.S. Simons, Jr., M. Pons, D.F. Johnson, E.B. Thompson (NCI), and R. Doherty (U. Md.)).

Application of NMR in Biochemical and Biological Systems

Basic information on the structure and molecular interactions of biologically important materials is of significance to biochemical and biological research. One of the most promising approaches in obtaining such information is through the use of nuclear magnetic resonance. The purpose of this project is to develop and apply nuclear magnetic resonance methods for studying molecular structures and molecular interactions in biochemical and biological systems.

Many of the biologically significant functions of metalloporphyrin-containing proteins are directly related to the interactions between the central metal and the porphyrin nitrogens and between the central metal and coordinating ligands. Such interactions should be very suitable for investigation by various NMR techniques. Although several recent communications have successfully demonstrated the use of N-15 NMR spectroscopy as a means of assessing structural information about porphyrin, metalloporphyrins, and related ring systems, relatively few investigations of these systems by means of metal nuclide NMR have been reported. However, a systematic study of N-15 and/or metal nuclide NMR spectroscopy of the metal-ligand and metal-porphyrin interactions in these molecules remains to be explored. We have made such systematic study of the effects of ligands on these interactions in doubly labeled cadmium meso-tetraphenylporphyrin [^{111}Cd-TPP($^{15}N_4$)] in order to examine the utility of these nuclei in obtaining useful information about these systems. To examine the effect of ligands on the Cd-111 or N-15 chemical shift as well as the Cd-N coupling constants, the ^{111}Cd-TPP($^{15}N_4$) was treated with a series of substituted pyridines. Our results indicate that the N-15 chemical shift is insensitive to the ligand effect and therefore it is not practical to use N-15 NMR as a tool for probing the interaction of ligand with metalloporphyrin. A relatively large Cd-111 chemical shift difference was observed for the series of ligands (∿17 ppm). The result suggests that Cd-111 NMR (and probably the other metal nuclides) is a better tool for probing the interaction of ligands with metalloporphyrins. A good correlation between the Cd-111 chemical shift and the pK values of the substituted pyridine ligands is observed. An extension of this work to other metal nuclides is in progress.

As a part of NMR studies on metalloporphyrin-containing molecules, we have undertaken a C-13 NMR study on the conformation and structure of hemoglobin which will be ^{13}C-labeled specific at the histidine residues. Work on the preparation of the ^{13}C-labeled hemoglobin is now in progress.

As a part of the project in the use of NMR for structural elucidation, various NMR methods have been employed successfully to assign the signals for H-4 and H-5 in alkylimidazoles and to probe the ring substituent effects of a series of anilines and anilinium ions.

Hemerythrin, a non-heme oxygen-carrying protein found in the erythrocytes of certain marine invertebrates, normally occur in an octameric form of molecular weight 108,000, and can be dissociated into the monomeric species in the presence of several organic mercurials. Each subunit contains two atoms of iron and can reversibly bind one molecule of oxygen. Oxidation of

either oxyhemerythrin or deoxyhemerythrin gives the iron (III) species, methemerythrin, which coordinates a number of simple anionic ligands to give a series of characteristic methemerythrin complexes.

We have made comparative NMR studies of the pH-dependent chemical shifts of histidine C-2 protons in the spectra of P. gouldii hemerythrin A and hemerythrin B, and T. pyroides myohemerythrin in their metazide forms in order to obtain local structural information of these proteins. By comparing their NMR spectra in the histidine resonances in hemerythrin A (His-82 and His-34) and one each in hemerythrin B (His-34) and myohemerythrin (His-57). The readily titrable His-82 (pK 6.5) in hemerythrin A and His-57 (pK 6.4) in myohemerythrin in the pH range 4 to 9.5 suggest that these histidines probably exposed near the surface of the protein. That His-34 in hemerythrin A and hemerythrin B can be observed only below pH 4.5 indicates that His-34 may be partially buried inside the protein or hydrogen-bonded to nearby amino acid residues. The results eliminate the possibility of His-32, His-34 and His-57 as ligands to the iron atoms and hence imply that a maximum number of five histidines are coordinated to the iron atoms.

We also examined the histidine C-2 proton titration behaviors of both octameric and monomeric methemerythrins with several anions (e.g., N_3^-, Cl^- and CN^-). The titration data for His-34 were found noticeably different between ligands such as N_3^- and Cl^- (or CN^-), and the pK_a values of His-34 were found considerably higher for the monomeric than the octameric methemerythrins. These findings tend to suggest that the local environment of His-34 is different for these methemerytrin ligand complexes and also suggest the involvement of His-34 in the intersubunit interactions. An extension of this work to other ligands is planned.

Neurotoxins from snake venoms are a group of small proteins which block the neuromuscular transmission at the post-synaptic membrane by high affinity binding to acetylcholine receptor and accordingly have been extensively studied in the field of neurochemistry. The toxins show a high degree of sequence homology and 13 amino acid residues are found to be invariant among more than 50 neurotoxins being investigated. The roles played by some of the amino acid residues in the binding to the receptor and in the lethal activity of the toxins have been the subject of considerable interest.

Basic information on the conformation and structure of the toxins in solution is of importance to the understanding of the nature of toxin-receptor interaction. Although the main chain conformation of these proteins has been elucidated in some detail, relatively few investigations on the local structure of the amino acid residues of the toxins have been reported. In order to gain understanding of the structure-toxicity relationship for the toxins, a possibility of using NMR to obtain the detailed structural information of these proteins has been recently explored. The preliminary results of NMR work on cobrotoxin and pelamitoxin have revealed some detailed structural information, including the microenvironments of His-4, Tyr-25 and His-32 of cobrotoxin and His-31 and His-54 of pelamitoxin. A detailed structural study of these toxins, including some chemical modifications, is now in progress (H.J.C. Yeh, D.F. Johnson, S. Chiang, F. Hayashi, Y. Takeuchi, K.L. Kirk, and L.A. Cohen).

The Development of Chemical Methods and Compounds for the Study of Biological and Medical Problems

The objective of this project is to make contributions to the investigation and solution of basic biological and medical problems by the application of chemical, physical, and biological methods.

Work is in progress on the study of cancer metastases in the mouse. The purposes of this study are (a) to increase our understanding of the biochemical mechanisms operating in the different stages of the formation of metastases, (b) to study the effects of chemicals on metastases and their development, and (c) to apply the knowledge obtained thereby to the design and synthesis of organic compounds of potential use in inhibiting the formation of metastases or selectively destroying them in human beings.

The findings of these studies may also contribute to the study of other biological and medical problems.

The effects of selected chemicals on metastases and their development in C57BL/6N are being studied following administration of cells of the T241 sarcoma or B16 melanoma intramuscularly, subcutaneously, or intravenously.

The population of cells in most malignant tumors is thought to be heterogeneous. If the subpopulation of the cells of a given tumor which accounts for the tumor's invasiveness and metastatic potency could be isolated from the primary tumor the study of such a cell line might reveal some new approaches to interference with the process of metastasis formation or to agents lethal to the metastatic cells. The isolation of a subpopulation of cells of high metastatic potential from the T241 sarcoma is being attempted by studying the binding of tumor cells to collagens I and IV and by soft agar techniques. The cell lines isolated from the B16 melanomas by I. Fidler are also being studied.

A neutral protease has been isolated from the highly invasive and metastic mouse sarcoma, T241, and partially purified. This enzyme preferentially degrades basement membrane collagen and may play a role in the invasiveness and metastatagenicity of the tumor. Experiments are in progress to determine whether the partially purified enzyme can provoke an immune response capable of interfering with tumor invasiveness and formation of metastases. If antibodies to the enzyme can be produced in another species, they will be useful in studying the distribution of the enzyme and its biological role. Using two types of assays employing cells of T241 sarcoma and B16 melanoma cell lines and media obtained from cell cultures, a correlation has been found between the incidence of spontaneous metastases and the kind of degradative activity displayed by this enzyme. If this enzyme does play a critical role in tumor invasiveness and metastasis formation, it may afford a route to interference with the process (M. Foltz and A. Liotta (NCI)).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19001-07 LC

PERIOD COVERED
October 1, 1978, to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immunochemistry and Reactions of Carbohydrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.P.J. Glaudemans	Chief, Sec. on Carbohydrates	LC	NIAMDD
Other:	M.K. Das	Visiting Fellow	LC	NIAMDD
	A.K. Bhattacharjee	Visiting Associate	LC	NIAMDD
	E. Zissis	Research Chemist	LC	NIAMDD
	L.G. Bennett	Staff Fellow	LC	NIAMDD
	A. Roy	Visiting Fellow	LC	NIAMDD

COOPERATING UNITS (if any)
K.J. Kwon-Chung (NIAID)

LAB/BRANCH
Laboratory of Chemisty

SECTION
Section on Carbohydrates

INSTITUTE AND LOCATION
NIAMDD, NIH, BETHESDA, MD 20205

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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

How specific are anti-carbohydrate immunoglobulins? What is the nature of the combining site? To answer these questions the interactions or homogeneous immunoglobulins with carbohydrate haptenic groups will be studied.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Homogeneous immunoglobulins can be obtained from plasma tumors. The study of their interaction with polysaccharide antigens will reveal much about the nature of action of antibodies and about their structure. Also, these systems are a general model for protein/carbohydrate interactions about which little is known to date.

Methods Employed: The usual techniques of chemistry and biochemistry are employed, such as affinity chromatography employing hapten-coupled sepharose columns, fluorescence titration, organic synthesis, gas-liquid chromatography, mass spectroscopy, etc.

Major Findings

1. On the nature of the binding of neutral ligands to immunoglobulins.

One of the most important and interesting areas in molecular biology concerns mutual attraction of complimentary molecules resulting in noncovalent associations and the myriad of cascading events which such associations trigger. In our Section we are increasingly involving ourselves in studying the nature of these associations. A number of D-galactopyranosides bearing aromatic substituents have been prepared, and their binding to immunoglobulin J539 (Fab') has been studied (see Table 1).

The data in Table 1 show that the protein only binds ligands that are β-glycosides of D-galactopyranose, i.e., the protein needs to recognize the β-D-galactopyranoside moiety in order to bind the ligand. Why do the 6-O-aromatically substituted β-D-galactopyranosyl residues show a higher affinity for the immunoglobulin combining-site compared to their unsubstituted analogs? We propose that the increased binding is due simply to the fact that these derivatives are more hydrophobic, and thus essentially less soluble in the aqueous phase, than their unsubstituted analogs.

The free energy of binding, ΔG, for the ligands is derived from the association constants Ka by the equation $\Delta G = -RT\ln Ka$. The values for these free energies of binding are listed in the 3rd column in Table 1. The process of binding a small, neutral ligand to a protein in aqueous solution may be regarded as starting with a hydrated ligand, stripping it of its hydration, at least partially, and then "removing" the solute from solution by "binding" it to the protein. The protein in the combining area will also be stripped of hydration, and the sum of these previously structured, water molecules will be randomly returned to the bulk solvent. The resulting increase in entropy could be the main driving-force for the binding of neutral, low polarity ligands.

When a small molecule binds to a large molecule (such as a protein), it loses translational and rotational degrees of freedom. That loss of entropy is the major effect unfavorable to association. For a molecule, such as that of a simple (oligo) saccharide, of mol. wt. <1000, this has been estimated to be ~70

kJ. If the overall favorable, free energy of binding is -18 kJ (as for methyl β-D-galactopyranoside), some process will have to yield about -88 kJ of free energy favorable to binding. The principle source of this energy could arise from the return to the solvent of surface molecules of water, due to mutual exclusion of solvent accessibility in the contact area between ligand and protein. The contribution to the free energy of this burial of previously accessible surface has been estimated to be ∿10.5 kJ/nm^2. By measurement, the surface area of a methyl hexopyranoside is, very approximately, 3.5 nm^2. Were this area to be enveloped by the protein surface in the combining area, the totally buried surface involved would be ∿7-9 nm^2; that could yield some 84 kJ of free energy favorable to binding, which makes up well for the loss of rotational and translational entropy already mentioned, and would give a negative ΔG of binding.

TABLE 1: Binding of IgA (Fab') J539 with galactose ligands.

Ligand	Ka (M^{-1})	Ka / KaMeGal	-ΔG (in kJ, with kcal in parentheses)
Methyl β-D-galacto-pyranoside (MeGal)	1.37×10^3	1	18 (4.27)
Methyl 6-O-benzoyl-β-D-galactopyranoside	2.89×10^3	2.10	19.89 (4.72)
Methyl 3-O-benzoyl-β-D-galactopyranoside			no change
Methyl 6-O-(p-methoxy benzoyl)-β-D-galactopyranoside	2.57×10^3	1.87	19.55 (4.64)
Methyl 6-O-(p-nitrobenzoyl)-β-D-galactopyranoside	?		very low change
m-Nitrophenyl β-D-galacto-pyranoside	1.52×10^5	110.9	29.75 (7.06)
p-Nitrophenyl β-D-galacto-pyranoside	1.06×10^5	77.37	28.86 (6.85)
o-Nitrophenyl β-D-galacto-pyranoside	4.0×10^4	29.19	26.42 (6.27)
Phenyl-β-D-galacto-pyranoside	1.30×10^4	9.48	23.60 (5.60)
Phenyl 1-thio-β-D-galacto-pyranoside	?		very low change
p-Aminophenyl 1-β-D-thio-galactopyranoside	9.35×10^3	6.82	22.79 (5.41)
p-Nitrophenyl α-galacto-pyranoside			no change
p-Nitrophenyl			no change

Some hydrogen bonding may very well occur in addition. This description is, of course, to a large extent, qualitative; however, there is evidence that hydrophobic bonding plays a significant role. For instance, the combining area of J539 does not display a larger number of potential, contact amino acids having a hydrophilic nature than any other surface area of the Fv immunoglobulin

fragment; thus, binding by hydrogen bonding to a ligand seems not to be selected for. From Table 1, it may be seen that 6-O-aromatically substituted glycosides show an increase in binding constants; this increase can come about in several ways. 6-O-Benzoyl-and 6-O-(p-methoxybenzoyl)-β-D-galactopyranoside have almost identical constants of association with J539 Fab'. It is unfortunate that affinity measurements with methyl 6-O-(p-nitrobenzoyl)-β-D-galactopyranoside were not possible. However, it was found that methyl 6-O-(p-nitrobenzoyl)-β-D-galactopyranoside and methyl β-D-galactopyranoside inhibit the precipitation of J539 immunoglobulin with pneumogalactan to exactly the same extent. These results indicate that a charge-transfer complex with the 6-O-aromatic groups and the proteins is unlikely, as there is no real difference when the phenyl ring is substituted with a methoxy or a nitro group. The increase in free energy of binding in going from methyl β-D-galactopyranoside to the corresponding 6-O-benzoyl and 6-O-(p-methoxybenzoyl) derivatives is in the range of 2.1 kJ (and thus small). Thus, it appears that the increase in binding of ligands that are substituted on O-6 with aromatic groups could be mostly due to their increased hydrophobicity, and not to any charge-transfer type of binding between the ligand and tyrosyl, tryptophanyl, or histidyl residues of the protein.

Turning now to the glycosides having an aromatic aglycon (see Table 1), it is, first of all, interesting that the binding affinity of phenyl β-D-galactopyranoside is essentially the same as that of Gal_2 and almost ten times that of methyl β-D-galactopyranoside; that is, there is an increase of ∿5.9 kJ of binding free-energy due to the presence of the phenyl glycosidic gourp. Consequently, if the aromatic substituent is the aglycon, its influence on the binding energy is much greater than when it is positioned as a peripheral group at the O-6. Phenyl β-D-galactopyranoside has its phenyl group in the same position as the second D-galactose residue in Gal_2. Thus, interaction of the protein with such glycosidic substitutents are much more likely than with substituents at O-6. Therefore, this larger increase in binding of phenyl β-D-galactopyranoside could be due to interactions between protein and ligand that are in addition to simple increases in binding free-energy derived from additional hydrophobicity of the ligand.

On inspection of the model of J539, it may be seen that there are three prominent, aromatic amino acid residues in the central part of the protein, namely, at position 93 of the light chain (L93) and positions 101 and 102 of the heavy chains (H101 and H102), all of which carry a tyrosine residue. It is a priori, quite possible, from the molecular dimensions of the ligand and of the complementary region of the protein, that (a) the (aglycon) phenyl ring may be capable of entering into π-π electron interactions, or (b) the nitro groups of m-, o-, and p-nitrophenyl β-D-galactoside could possibly enter into hydrogen-bond formation with these tyrosine residues, as substitution on the ring with the nitro group significantly enhances the binding to immunoglobulin J539 (see Table 1), or both. The finding that, of the three, the o-nitro glycoside shows the weakest binding increase may be rationalized by the fact that inspection of the model of this ligand shows quite clearly that an internal hydrogen-bond can occur between this nitro group and the 2-hydroxyl group of the sugar, thus partially neutralizing the effect of the ring substitution (M.K. Das, E. Zissis and C.P.J. Glaudemans).

2. On the nature of the binding of charged ligands to immunoglobulins.

A system that has received considerable attention is the antigen-antibody system involving the choline or phosphorylcholine (PC) hapten. Thirteen murine anti-PC myeloma antibodies have been described and studied to varying degrees thus far by others and ourselves. A complete three-dimensional structure of one immunoglobulin, M603, has been determined to a 3.1 Å resolution from x-ray diffraction data by D. Davies and his groups here at NIH. From crystallographic studies with the phosphorylcholine ligand present in the binding site, the identities and relative locations of the amino acid side chains that form the combining site are known and have been summarized by the above mentioned workers as follows: "Electrostatic interactions, hydrogen bonding, and van der Waals forces contribute to the interaction between phosphorylcholine and the M603 protein. The positively charged trimethylammonium moiety of the hapten interacts with the acid groups of Glu-23H and Glu-59H. The negatively charged phosphate group interacts with the positively charged guanidinium of Arg-52H and possibly also with the side chain of Lys-54H. In addition, one oxygen of the phosphate is hydrogen bonded to the phenolic OH of Tyr-33H and another to the guanidinium of Arg-52H. There are extensive van der Waals contacts between hapten and the side groups of Tyr-33H and Trp-104aH and an as yet unidentified residue at position 96 of the light chain".

We have used H-8 instead of M603, however the important hapten-contacting residues (Tyr-33H, Glu-35H, Arg-52H, Lys-54H, and Glu-59H) are the same in both proteins. Moreover, of the three hypervariable (hv) loops of the heavy chain, the first and second have exactly the same number of amino acid residues and thus probably the identical conformation. The third heavy chain hv loop, while having one amino acid less than M603, has the contact residue (Trp-104aH) in the exact same position as in M603 since it is only two residues removed from the framework of the variable region.

Results of our binding assays permit an assessment of the apparent lack of specificity of the antibody, as manifested by its ability to bind a variety of compounds. Also the apparent flexibility of the antibody combining site as manifested by the ability to bind compounds of varying molecular size will be discussed. Finally, we will describe some of the apparent steric and electronic requirements that ligands must possess if they are to bind in the combining site where phosphorylcholine and "choline-like" compounds bind.

Our interest in the binding of choline and similar analogues to specific protein was stimulated by the discussion of Padlan of the binding of phosphorylcholine to the homogeneous immunoglobulin M603. Little reference was made to the importance of the three methyl groups around the quaternary ammonium nitrogen. It is obvious that these methyl groups must be essential to the binding because if they are absent the ligand becomes phosphorylethanolamine, a molecule whose affinity for M603 is known to be negligible. Also, since ethanolamine is positively charged at physiological pH, it seemed that the importance of the ionic attraction between the protein's glutamic acid residues and the hapten's quaternary ammonium was over-emphasized. Analogues were obtained in which the charged

nitrogen was replaced by a neutral carbon. Thus, phosphorylcholine was compared with 3,3-dimethylbutyric acid (Table 2). 3,3-Dimethylbutanol was found to be very poorly soluble, but even under conditions where the aqueous solution containing the protein was saturated with the ligand no binding was detected. The steric arrangement of the three methyl groups surrounding either the charged nitrogen or betaine or the neutral carbon of 3,3-dimethylbutyrate is approximately equivalent. The only significant difference is that betaine bears a positive charge, whereas the other does not. The binding data showed that whereas the betaine bound the antibody with a Ka of 3.6×10^3 M^{-1}, the 3,3-dimethylbutyric acid showed no detectable binding and was unable to inhibit PC binding.

Results of the binding assay with 3,3-dimethylbutanol phosphate as the ligand were ambiguous. Data points did not provide a characteristic saturation curve. Titration of H-8 Fab' with phosphorylcholine in the presence of 0.1 M 3,3-dimethylbutanol phosphate resulted in a much lower fluorescence enhancement than expected, suggesting that the binding of PC was being inhibited in a noncompetitive manner. One possible explanation of this behavior is that the 3,3-dimethylbutanol phosphate possesses sufficient detergent-like properties so as to partially denature the protein, thus preventing or inhibiting binding of PC. Nevertheless, binding assays for betaine and 3,3-dimethylbutyric acid clearly suggested that the methyl groups of the PC ligand per se are not directly involved in binding and that any van der Waals attractions contributed to binding of the PC ligand are probably minor contributions to the overall binding affinity. Thus, in the case of the homogeneous H-8 Fab', the ionic attraction between negatively charged groups of the protein's combining site and the positively charged portion of the ligand appears to be essential for significant binding to occur. Not surprisingly, we found that not only should the ligand be charged, but it should be positively charged if it is to bind in the same combining site as choline. Thus, while positively charged tetraphenylarsonium binds H-8 nearly as well as choline itself (Table 2), negatively charged tetraphenylboron showed no detectable binding. The stereochemistry of these two molecules should be quite similar.

For the interaction between charged species, in aqueous environments, the distance of charge separation is important, but equally important is the dielectric value and the pH in the immediate vicinity of each species. The microenvironment of areas in the combining site of a protein may vary tremendously with respect to dielectric constant and pH. The importance of such parameters is obvious in a noncovalent association such as the interaction of choline and H-8 Fab'.

The dielectric nature of the microenvironment of the binding site may differ substantially from that of the bulk solvent. Protein microenvironments may vary from a nonpolar dielectric of about 2 to a polar dielectric of about 78. A relatively small change in the dielectric of the combining pocket of the protein may be reflected in a rather substantial change in the observed equilibrium constant of association between the protein and ligand, and we propose this as a possible explanation for the observed binding phenomena between H-8

Fab' and the ligands listed in Table 3; binding studies showed the ammonium ion to have little affinity for the protein's combining site. As the hydrogens of the ammonium ion are replaced by methyl groups (monomethyl, dimethyl, trimethyl, and tetramethyl), the ammonium ion shows progressively more affinity for the combining site, and the tetramethylammonium ion has a greater affinity than does choline itself (5.8×10^3 and 2.7×10^3 M^{-1}, respectively). Replacement of the hydrogens on the ammonium ion by methyl groups not only allows for increased van der Waals contacts between the combining site of the protein and the ligand, but could provide a mechanism whereby more water is excluded from the vincinity of Glu-35H and Glu-59H in the combining site, both of which have been implicated in ligand binding. As we have previously discussed, increased surface area available for van der Waals contacts is probably not terribly important. The contribution to binding would be twofold if more bound water could be displaced from the combining site: (a) lowering the dielectric of that microenvironment and providing for a stronger ionic attraction and (b) returning structured water molecules to the bulk solvent, causing an entropy change favorable to binding. The concepts described above could qualitatively answer the question as to why phosphorylethanolamine and other aliphatic amines, for example, may be unable to bind in the choline binding site of H-8 since in the absence of sufficiently bulky hydrophobic groups surrounding the charge enough water is not displaced from the combining site.

TABLE 2: Association equilibrium constants, free energy of binding, and observed maximum fluorescence change of H-8 Fab' upon binding ligands possessing a charged quaternary ammonium and analogues not possessing a quaternary ammonium group.

Ligand	Keq (M^{-1})	ΔG (kcal/mol)	ΔF_{max} (%)
$H_3C^+N(CH_3)_2CH_2CH_2OH$ (choline)	2.7×10^3	-4.67	+28.2
$H_3CC(CH_3)_2CH_2CH_2OH$ (3,3-dimethylbutanol)	nbd[a]		0
$H_3C^+N(CH_3)_2CH_2COO^-$ (betaine)	3.6×10^3	-4.84	+22.4
$H_3CC(CH_3)_2CH_2COO^-$ (3,3-dimethylbutyrate)	nbd		0
$H_3C^+N(CH_3)_2CH_2CH_2OP(=O)(O^-)_2$ (phosphorylcholine)	6.8×10^5	-7.93	+35.4
$H_3CC(CH_3)_2CH_2CH_2OP(=O)(O^-)_2$ (3,3-dimethylbutanol phosphate)	b		quenching
Ph_4As^+ (tetraphenylarsonium)	1.2×10^3	-4.19	-33.2
Ph_4B^- (tetraphenylboride)	nbd		0

[a]nbd=no binding detected. [b]See text for explanation.

TABLE 3: Association equilibrium constants, free energy of binding, and observed maximum fluorescence change of H-8 Fab' upon binding ammonium ions bearing increasing degrees of methyl substitution.

Ligand	Keq (M^{-1})	ΔG (kcal/mol)	ΔF_{max} (%)
NH_4^+	nbd		0
$(CH_3)NH_3^+$	nbd		0
$(CH_3)NH_2^+$	$4.0x10^1$	-2.18	+7.0
$(CH_3)_3NH^+$	$3.5x10^2$	-3.46	+9.7
$(CH_3)_4N^+$	$5.8x10^3$	-5.12	+25.1

nbd=no binding detected.

TABLE 4: Association equilibrium constants, free energy of binding, and observed maximum fluorescence change of H-8 Fab' upon binding primary amines and symmetrical quaternary ammonium ligands bearing hydrophobic or hydrophilic substitutents.

Ligand	Keq (M^{-1})	ΔG (kcal/mol)	ΔF_{max} (%)
$H_3CCH_2NH_3^+$ (ethylamine	10^a		0
$H_3CC(CH_3)_2NH_3^+$ (1,1-dimethylethylamine)	$1.4x10^3$	-4.28	+24.3
$HOH_2CC(CH_2OH)_2NH_3^+$ [tris(hydroxymethyl) aminomethane]	nbd		0
$(H_3CCH_2)_4N^+$ (tetraethylammonium)	$2.8x10^3$	-4.69	+22.6
$(HOCH_2CH_2)_4N^+$]tetrakis(hydroxyethyl) ammonium]	$2.7x10^1$ b	-1.95	
$(H_3CCH_2CH_2)_4N^+$ (tetrapropylammonium)	$2.5x10^2$	-3.20	+22.8

[a]At sufficiently high concentrations (0.1 M) of ethylamine, some inhibition of choline was detected. An accurate assessment of the Keq was not possible, however. [b]Determined by inhibition of choline binding. nbd=no binding determined.

The nature of the groups surrounding the charged nitrogen or quaternary ammonium should be hydrophobic and not hydrophilic if significant binding is to occur. The mere presence of bulky groups around the charged nitrogen may facilitate binding, but hydrophobic groups appear to be much better than hydrophilic groups at promoting binding. Binding data on some representative ligands is shown in Table 4. Ethylamine showed only very slight affinity for the choline combining site. At high concentrations of ethylamine (0.1 M) some inhibition of choline binding was observed, but the data suggested an affinity constant for

ethylamine of less than 10 M^{-1}. When the two hydrogens on the C-1 of ethylamine were replaced by methyls to give 1,1-dimethylethylamine (tert-butylamine), the affinity for the choline binding site on H-8 increased dramatically (Table 4). Minimizing the importance of van der Waals contacts receives support from the observation that hydrophilic groups surrounding the positively charged nitrogen are less effective than hydrophobic groups in promoting binding, even though the opportunity for van der Waals contacts is unchanged in comparison to the situation in which hydrophobic groups surround the charge. Thus, when one hydrogen on each of the three methyls of 1,1-dimethylethylamine is replaced by a hydroxyl group to give tris(hydroxymethyl)aminomethane, the binding affinity drops by as much as three orders of magnitude from 1.4×10^{3} M^{-1} to a state in which no binding was detected with the tris ligand (Table 4). The same effect was observed, although not dramatically, with the quaternary ammonium compounds, tetraethylammonium, tetrakis(hydroxyethyl)ammonium, and tetrapropylammonium (Table 4). When a hydrogen on each of the four methyls of tetraethylammonium was replaced by a hydroxyl group, the binding affinity dropped by about two orders of magnitude from 2.8×10^{3} to 2.7×10^{1} M^{-1}. Some of this decrease in affinity could a priori be steric in nature in that the hydroxyl group is larger than a hydrogen, but it is not larger than a methyl, and as is evident from the binding affinity of tetrapropylammonium, the presence of a hydroxyl simply reduced the binding affinity regardless of the steric factors involved. Also, tetraphenylarsonium and -phosphonium bind to H-8.

The protein's apparent ability to accommodate charged ligands substantially larger than tetramethylammonium was also of particular interest, especially in view of the proposed limitations of the combining site area as discussed in some earlier investigations by Drs. Davies and Padlan at NIH. Table 4 lists the binding equilibrium constants of a series of symmetrical quaternary ammonium compounds of increasing size, along with the maximum molecular radius. The most likely conclusion is that the antibody combining site can possess a great deal of flexibility in order to accommodate ligands of such different sizes.

Two ligands which are much more defined in their steric conformation are tetraphenylarsonium and benzyltriphenylphosphonium. Due to the rigidity of the aromatic rings, the molecular volumes of these ligands are much easier to calculate. Thus, they occupy 6-7 times the volume of tetramethylammonium, but their binding affinities are not a great deal less than that of tetramethylammonium. We conclude that the H-8 antibody is capable of substantial flexibility in the region of its combining site for choline. (L.G. Bennett and C.P.J. Glaudemans).

3. Structural studies on the capsular polysaccharides of Cryptococcus neoformans Types C and D.

a. Type C.

In an earlier report we have discussed the structure of the capsular polysaccharide from Cryptococcus neoformans serotype C. We showed that the main chain was composed of α 1→3 linked mannose units and that the xylose and glucuronic acid were present as single unit branches on the mannose units. The

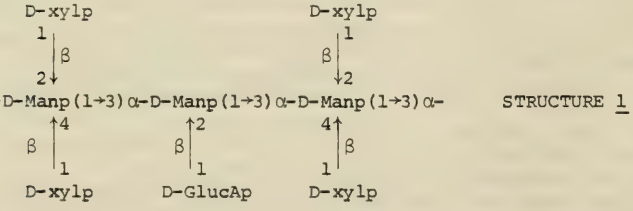
location of the glucuronic acid residue was shown to be either on a mannose residue bearing no other substituent or on a mannose unit bearing one xylose unit as an end group. Further experiments have now been performed which show that the latter assignment for the location of glucuronic acid is correct.

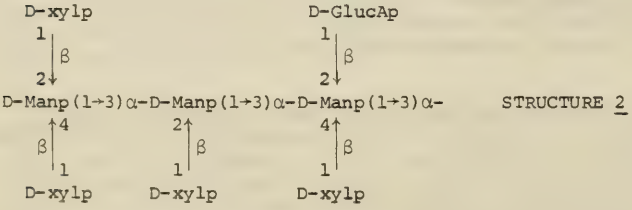
Hydrolysis of the methylated polysaccharide and conversion of the constituent sugars into alditol acetates followed by analysis by g.c. and g.c./m.s. showed the presence of 2,3,4-tri-O-methyl xylose, 4,6-di-O-methyl-mannose and 6-O-methyl mannose in the approximate molar proportion of 4:1:2.

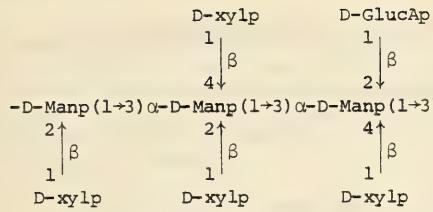
The alkaline degraded, remethylated polysaccharide, on similar analysis showed the presence of 2,3,4-tri-O-methyl xylose, 4,6-di-O-methyl mannose, 2,6-di-O-methyl mannose, and 6-O-methyl mannose in the approximate molar ratio of 4:1:1:1. Thus in this case 1 mole of 6-O-methyl mannose has been replaced by 1 mole of 2,6-di-O-methyl mannose.

Considering the two alternative structures that we proposed in our earlier report (shown below), it is clear from the results of the alkaline degradation that structure 2 shows the correct location of the glucuronic acid units so that their removal results in the formation of 2,6-di-O-methyl mannoses upon remethylation.

It is still possible, however, to change the sequence of sugars in structure 2 and get the same results, as shown in structure 2a. Thus the studies obtained from our studies agree well with either structure 2 or 2a (A.K. Bhattacharjee, K.J. Kwon-Chung and C.P.J. Glaudemans).







STRUCTURE 2a

b. Type D.

The purified polysaccharide of C. neoformans serotype D was obtained by ion-exchange chromatography on DEAE-cellulose and gave a homogeneous peak on gel chromatography on sepharose 6B. The Kav was 0.09, indicating a very high molecular weight (ca 8×10^5). The purified polysaccharide had $[\alpha]_{589}^{20}$ +32°. Estimation of O-acetyl showed the presence of 10.3% O-acetyl. The polysaccharide gave a single band in immunoelectrophoresis and moved towards the anode.

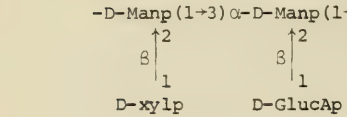
Complete hydrolysis of the polysaccharide followed by chromatography in solvents A and B showed the presence of three sugars having mobilities identical to mannose, xylose and glucuronic acid. The presence of glucuronic acid was confirmed by analysis of the carboxyl-reduced polysaccharide, when glucose was obtained instead of glucuronic acid.

Two acidic oligosaccharides were isolated in yields of 10 mg and 3 mg respectively. Oligosaccharide 1 was identified as 2-O-β-D-glucuronopyranosyl-D-mannose by hydrolysis, borohydride reduction followed by hydrolysis and by methylation analysis. The optical rotation of the oligosaccharide 1 $[\alpha]_{589}^{20}$ -33° (c. 1.0 in water) indicated that the glucuronic acid was lined β to the mannose reducing unit. This was further confirmed by the release of glucuronic acid on treatment with β-glucuronidase. Oligosaccharide 2 was a trisaccharide composed of two mannose residues and one glucuronic acid residue.

Chromium trioxide oxidation of the polysaccharide resulted in the loss of 80% of xylose residues (in two hours) showing that these units were β-linked in the polysaccharide, and that the mannose was α-linked.

Periodate oxidation of the polysaccharide resulted in the uptake of 1.0 mole of periodate (constant after 24 hours). Periodate oxidation followed by borohydride reduction and mild hydrolysis (Smith degradation) gave rise to a periodate-resistant polysaccharide which was recovered by dialysis and freeze drying. This material had $[\alpha]_{589}^{20°C}$ +115° (c. 0.5 in H_2O). Hydrolysis of this polysaccharide gave only mannose. Methylation followed by hydrolysis, conversion into alditol acetates and analysis by g.c. and g.c./m.s. showed the presence of 2,4,6-tri-O-methyl mannose as the major component (93%), indicating that the periodate resistant backbone was a 1→3 linked mannan. However there was also a minor component (7%) which was found to be 4,6-di-O-methyl mannose. If demethylation is ignored, this indicates that 1 in 15 mannose units in the mannan backbone carries a branch at C2.

The following structural formula is proposed for the capsular polysaccharide here investigated:



Immunodiffusion test in agar gel using rabbit antisera to whole cells of C. neoformans serotype C and D showed that the sera gave sharp bands against type-specific polysaccharides. The anti type D serum was only weakly cross reactive with type C polysaccharide, probably because C polysaccharide has few if any O-acetate groups. However anti type C serum reacts strongly with type D polysaccharide. Carboxyl-reduced polysaccharide type D reacts very weakly with anti type C serum thus showing that the carboxyl groups are strong determinants for this antiserum. Carboxyl-reduced and de-O-acetylated polysaccharide type D gave a very weak or no precipitin band against anti type D serum showing that both the carboxyl and the O-acetyl groups were important determinants of antigenic specificity in this polysaccharide. Smith degraded polysaccharide failed to show any precipitin band against either sera indicating the absence of precipitating antibody against the mannan backbone (A.K. Bhattacharjee, K.J. Kwon-Chung and C.P.J. Glaudemans).

4. The interaction of dextran with an immunoglobulin binding terminally linked glucose only.

Bivalent immunoglobulins bind ligands in their combining sites, each one of which can vary from 1.4 to 3.0 nm in length. Polysaccharide antigens bearing multiple, identical determinants generally bind many immunoglobulin molecules per single polysaccharide chain, each determinant of ∿2.0 nm being potentially capable of association with an immunoglobulin combining-site. Indeed, the resulting crosslinking of polysaccharides by immunoglobulins, leading to precipitation of the complex, has been extensively investigated. Thus, it is unusual for serum immunoglobulins to bind to a polymeric, multideterminant antigen while not precipitating with it. Cisar, Kabat, Dorner and Liao observed that immunoglobulin W3129 maximally binds to isomaltopentaose, but that the immunoglobulin does not precipitate with a synthetic, linear α-D-(1→6)-linked dextran of molecular weight 36,500, whereas it does precipitate with more highly branched (1→6)-α-D-dextrans.

We have investigated this problem by using a different approach. Our results are equally compatible with either of two possibilities: (a) IgA W3129 binds strongly to the nonreducing-terminal D-glucopentaose segments of the (1→6)-α-D-dextran, or (b) the IgA binds weakly to intercatenary segments of D-glucopentaose in the (1→6)-α-D-dextran. Combined with the precipitin data previously obtained, our results fully confirm the conclusions of Cisar et al.

We have measured the affinity constant (Ka) for the association between protein W3129 Fab' and a linear, α-D-(1→6)-linked dextran of molecular weight ∿36,000. The dextran was first purified by chromatography on Sephadex and the central (high molecular weight) part of the elution peak was taken, in order to avoid any possibility of contamination with oligosaccharides, as it had already been shown that the immunoglobulin is capable of maximally binding a pentasaccharide fragment.

We wished to determine whether or not a (nonreducing) terminal D-glucosyl group is a requirement for binding of W3129 to a (1→6)-α-D-dextran. If such is the case, then equimolar amounts of dextran of high molecular weight (M.W. 36,000; 500 μM = 18 mg/mL) and pentasaccharide necessary to saturate the available combining-sites of immunoglobulin W3129 (pentasaccharide M.W. 828; 500 μM = 0.41 mg/mL) have equal numbers of nonterminal D-glucosyl residues per unit volume, and therefore should exhibit the same, concentration-dependent, site saturation of Ig W3129 despite the fact that the dextran solution contains 44 times as much D-glucose as an equal volume of the pentasaccharide solution.

TABLE 5: Equilibrium-binding constants between W3129 Fab' and specific ligands.

Ligand	Ka			
	FT[a]	FT[a,b]	ED[a,b]	FI[a,b]
Methyl α-D-glucopyranoside	1.8×10^3	1.4×10^3	9.4×10^2	8.0×10^2
Isomaltopentaose	1.65×10^5	1.7×10^5	1.9×10^5	1.7×10^5
Dextran (M.W. ∿36,000)	2.0×10^5			

[a]Abbreviations: FT, fluorescence titration; ED, equilibrium dialysis; and PI, precipitation inhibition. Equilibrium-binding constants have the units M^{-1}.
[b]Data from Cisar et al. for intact immunoglobulin W3129.

The equilibrium constants (Ka) in Table 5 were calculated by using a molecular weight of 36,000 for the dextran and of 828 for the pentaose, yielding almost identical Ka values. This shows that the concentration of available ligand is the same for the solution containing 0.41 mg of pentasaccharide per mL and the dextran solution containing 18 mg of polysaccharide per mL.

Thus, it appears that the chain of α-D-(1→6)-linked glucosyl residues beyond the 5 units at the nonreducing end of the dextran provides few or no additional binding sites for Ig W3129 Fab', and thus, that this protein is only capable of binding terminal D-glucosyl groups. This confirms the data of Cisar et al. In addition, it may be seen from Table 5 that the results from the method of ligand-induced, fluorescent titration agree well with those from other methods of determination of Ka, and we have also reconfirmed that Fab' fragments bind to their ligands with the same affinity as that of the intact immunoglobulin. (L.G. Bennett and C.P.J. Glaudemans).

Publications

Glaudemans, C.P.J., Das, M.K., and Vrana, M.: Homogeneous murine immunoglobulins with anti-carbohydrate specificity. Methods in Enzymol. 50 C: 316-323, 1978.

Manjula, B.N., and Glaudemans, C.P.J.: Affinity of horse anti-phosphorylcholine antibodies for phosphorylcholine containing polysaccharides. Contribution of the polysaccharide backbone. Immunochem. 15: 269-271, 1978.

Vuk-Pavlovic, S., Blatt, Y., Lancet, D., Pecht, I., and Glaudemans, C.P.J.: Hapten linked conformational equilibria in immunoglobulin XRPC24 and J539 observed by chemical relaxation. Biophysical J. 23: 161-174, 1978.

Bhattacharjee, A.K., and Glaudemans, C.P.J.: The binding of 5-dimethyl-1-naphthalene sulfonyl (dansyl) derivatives of D-galactose saccharides to several Ig's capable of binding β(1→6)linked D-galactopyranan. Carbohyd. Res. 67: 536-540, 1978.

Bhattacharjee, A.K., and Glaudemans, C.P.J.: On the structure of the capsular polysaccharide from Cryptococcus neoformans serotype C. Immunochem. 15: 673-679, 1978.

Streefkerk, D.G., Manjula, B.N., Glaudemans, C.P.J.: An interpretation of the apparent dual specificity of some murine myeloma immunoglobulins with inulin binding activity. J. Immunol. 122: 537-541, 1978.

Das, M.K., Streefkerk, D.G., and Glaudemans, C.P.J.: The binding of inulo-oligosaccharides to homogeneous immunoglobulins E109 and A47N. Mol. Immunol. 16: 97-100, 1979.

Bennett, L.G. and Glaudemans, C.P.J.: The affinity of an α(1,6)linked linear D-glucopyranan (dextran) for homogeneous immunoglobulin A W3129. Carbohyd. Res. 72: 315-319, 1979.

Das, M.K., Zissis, E. and Glaudemans, C.P.J.: The binding of aromatic derivatives of galactan to IgA Fab' J539. Carbohyd. Res. 73: 235-244, 1979.

Bhattacharjee, A.K., Kwon-Chung, K.J. and Glaudemans, C.P.J.: The structure of the capsular polysaccharide from Cryptococcus neoformans serotype D. Carbohyd. Res. 73: 183-192, 1979.

Potter, M., Mushinski, E.B., Rudikoff, S., Glaudemans, C.P.J., Padlan, E. and Davies, D.R.: Structural and genetic basis of idiotypes in galactan binding myeloma protein. Annals of Immunol., Inst. Pasteur 130 C: 263-271, 1979.

Bennett, L.G., Glaudemans, C.P.J.: Binding studies with antibodies having phosphorylcholine specificity and fragments derived from their homologous streptococcus pneumoniae type 27 capsular polysaccharide. J. Immunol. 122: 2356-2362, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19200-29LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biological Testing for Antinociceptive Activity, Acute Toxicity and Dependence Liability

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. E. Jacobson	Research Chemist	NIAMDD-LC
OTHER:	E. L. Atwell	Medical Biology Technician	NIAMDD-LC

COOPERATING UNITS (if any)
Department of Pharmacology of the University of Michigan and the Medical College of Virginia, and the Committee on Problems of Drug Dependence, Inc.

LAB/BRANCH
Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES xx☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Animal tests are used to determine the antinociceptive activity of potential analgesic agents. Acute toxicities have, also, been determined on a number of compounds with disparate biological activities. About 40 to 50 compounds have been sent to our cooperating units for further investigation; others have been sent to NCI for screening.

Various mouse assay procedures are utilized for initial exploration of the analgesic potential of molecules. This information is necessary to determine a starting point for their further investigation, at the Medical College of Virginia and the University of Michigan. Thus, compounds which are of interest are examined, under the auspices of the Committee on Problems of Drug Dependence, Inc., in four rodent antinociceptive assays, in a rodent assay for narcotic antagonist activity, and in a series of tests in the rhesus monkey. The binding affinity of these compounds to the opiate receptors in rat brain homogenates, electrically stimulated guinea pig ileum and mouse vas deferens, is also determined. These data provide objective information about the pharmacological action and possible side-effects of potential analgesics before human clinical studies are undertaken.

Acute (7-day) toxicity studies, in mice, of colchicine-like compounds help provide initial information which, coupled with tubulin binding data, allow us to choose compounds for further (NCI) screening.

Publications:

1. Atwell, L., and Jacobson, A. E.: The Search for Less Harmful Analgesics. Lab. Animal 7: 42-47, 1978.

2. Jacobson, A. E.: Biological Testing Program of the Committee on Problems of Drug Dependence, Inc. III. 1979 Annual Report. In: Problems of Drug Dependence, 1979. Washington, D.C., NIDA Monograph, 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19201-05LC

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Preparation, Receptor Binding Affinity, and Pharmacological Evaluation of N-Cyanoalkyl Opioids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. E. Jacobson	Research Chemist	NIAMDD-LC
OTHER:	K. C. Rice	Research Chemist	NIAMDD-LC
	J. Reden	Guest Worker	NIAMDD-LC
	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC

COOPERATING UNITS (if any)

Laboratory of General and Comparative Biochemistry, NIMH, ADAMHA; Departments of Pharmacology, University of Michigan and Medicinal College of Virginia; Committee on Problems of Drug Dependence, Inc.

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NIAMDD, NIH, Bethesda, Maryland 20205

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0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Temporarily discontinued.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19202-06LC

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Synthesis and Evaluation of Potential CNS, Antiinflammatory and Anticancer agents.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. C. Rice	Research Chemist	NIAMDD-LC
OTHER:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC

COOPERATING UNITS (if any)

Medical College of Virginia (E. L. May, L. . Harris, W. L. Dewey and M. D. Aceto); Pennsylvania State University, York Campus (E. A. Harrison).

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NIAMDD, NIH, Bethesda, Maryland 20205

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0.4	0.4	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new base induced ring cleavage was observed in the 2-aryl-3-hydroxy-thieno[2,3-b]quinoline-1,1-dioxide series. Several of these compounds were inactive as antineoplastic agents.

The 2-aryl-3-hydroxythieno[2,3-b]quinoline-1,1-dioxides which we reported earlier as a novel class of phosphodiesterase inhibitors have been shown to undergo an interesting base induced ring cleavage. When sodium methoxide in methanol is used as the base, this new reaction results in loss of sulfur dioxide from the parent molecule and formation of the corresponding benzyl 3-(2-methoxyquinolyl) ketone. This reaction appears to proceed with loss of sulfur dioxide from an intermediate sulfinic acid which is formed by attack of methoxide ion on the parent molecule followed by ring opening. A series of 2-aryl-3-hydroxythieno[2,3-b]quinoline-1,1-dioxides submitted for screening at the request of NCI were inactive as antineoplastic agents.

Publications:

Iijima, I. and Rice, K. C.: An Efficient Synthesis of 3-aminoisothizolo [5,4-b]quinoline from Quinoline. J. Heterocyclic Chem., 15, 1527-1528 (1978).

Harrison, E. A. and Rice, K. C.: Novel Alkaline Ring Cleavage of 2-Phenyl-3-hydroxythieno[2,3-b]quinoline-1,1-dioxide A Potent Inhibitor of Cyclic AMP Phosphodiesterase. J. Org. Chem., in press (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19208-04 LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Synthesis and Pharmacology of (+)-Antipodes of Narcotic Agonists and Antagonist

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. C. Rice	Research Chemist	NIAMDD-LC
OTHER:	A. Brossi	Chief, Medicinal Chem.	NIAMDD-LC
	A. E. Jacobson	Research Chemist	NIAMDD-LC
	J. Minamikawa	Visiting Fellow	NIAMDD-LC

COOPERATING UNITS (if any)
NIMH (W. Klee); Medical College of Virginia (L. S. Harris, E. L. May, W. L. Dewey, M. D. Aceto, University of Michigan (H. H. Swain); New York State Research Institute for Neurochemistry and Drug Addiction (Y. Jacquet).

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NIAMDD, NIH, Bethesda, Maryland 20205

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0.8	0.8	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been completed.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19212-03LC

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Practical Total Synthesis of Codeine and Other Important Opium Alkaloids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	K. C. Rice	Research Chemist	NIAMDD-LC
	W. Ripka	Guest Worker	NIAMDD-LC
	F. Hsu	Staff Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

University of Wisconsin (D. Pearlman), Oregon State University (J. White)

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NIAMDD, NIH, Bethesda, Maryland 20205

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0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Additional efforts to find conditions for a high yield conversion of reticuline (or derivative) to salutaridine (or derivative) have not been successful, however N-acyl-4-methoxy-N-norreticuline derivatives, which can be readily converted to either the pavinan or isopavinan carbon skeleton are now readily available from this work. A simple, high yielding optical resolution of (+)-N-norreticuline has been developed. Aporphine formation has been largely suppressed in the phenolic oxidation of N-carbethoxy-N-norreticuline to N-carbethoxy-N-nor-salutaridine. Efforts at total synthesis of codeine and morphine are now concentrated on a modified Grewe approach which involves 4-hydroxy-6-keto-17-methylmorphinan as a key intermediate.

PHS-6040
(Rev. 10-76)

With a variety of N-substituted-N-norreticuline derivatives now readily available as a result of our previous work, we have continued to study the phenolic oxidation of these substances with the goal of finding conditions which afford the corresponding salutaridine derivative in high yield. Although we have not been successful in vastly improving this key step in the total synthesis of codeine by the biomimetic route, a number of interesting results have been obtained in the area of phenolic oxidation. For example, the troublesome side reaction involving aporphine formation in the oxidation of N-carbethoxy-N-norreticuline with vanadium oxytrifluoride has been largely suppressed and the yield of 6'-bromo-N-carbethoxysalutaridine is now 30%. Oxidation of several N-acyl-N-norreticuline derivatives with DDQ in methanol gave the corresponding 4-methoxy compounds by a reaction which probably involves methanol addition to an intermediate quinone methide. In contrast to the 4-hydroxy-1-benzyltetrahydroisoquinolines, the 4-methoxy derivatives are stable compounds and can be readily converted into either pavinan or isopavinan carbon skeleton. We have accomplished a very simple, high yielding optical resolution of (±)- N-norreticuline which gives both enantiomers in nearly 90% yield and have utilized (-)-N-norreticuline to prepare a number of racemic and natural pavinan and isopavinan alkaloids as described in the accompanying report.

Our current approach to the total synthesis of morphine and codeine involves a modification of the Grewe approach reported by a Dutch group. This approach may afford 4-hydroxy-6-keto-17-methyl-morphinan as a key intermediate. We have already converted this material (obtained by chemical degradation of natural morphine) to 3-desoxydihydromorphine and are now concentrating on the conversion of the latter to dihydromorphine and on a practical synthesis of (-)-4-hydroxy-6-keto-17-methyl-morphinan.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19214-03LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Direct and Sequential Removal of Phenolic Oxygen Functionalities in Alkaloids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	J. Minamikawa	Research Fellow	NIAMDD-LC
	K.C. Rice	Research Chemist	NIAMDD-LC

COOPERATING UNITS (if any)

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Aromatic methoxy groups can be cleaved selectively in the presence of aromatic methylenedioxy groups. This, together with the removal of the newly formed phenol as its tetrazolylether by catalytic hydrogenation, has been utilized to prepared some rare aporphine alkaloids.

PHS-6040
(Rev. 10-76)

Whereas aromatic methylenedioxy groups, present in many alkaloidal systems, can easily be cleaved selectively with a variety of methods (Lewis acids, alkali metal in liquid ammonia), the reversal of this reaction has not been possible until now. It has now been found that treatment of methoxy-methylenedioxy substituted models with trimethylsilyl iodide in boiling quinoline affords the respective phenolic compounds with an intact methylenedioxy system. This method could not be directly applied to alkaloids with a basic funtionality. Methyl iodide formed during the reaction is trapped by the basic nitrogen function of the alkaloid to afford quaternary salts and reaction products thereof. It has been found that this side reaction can be considerably suppressed by the addition of strong bases such as DABCO, affording the expected phenolic materials in reasonable yield. The aporphine alkaloid (+)-laureline could thus be converted into (+)-mecambroline and removal of the phenolic group formed by catalytic deoxygenation of its tetrazolyl ether afforded (+)-roemerine. Reduction of (+)-bulbocapnine phosphate with lithium in liquid ammonia opened the methylenedioxy groups and eliminated two oxygenated functions to afford directly and in high yield (+)-2,10-dimethoxyaporphine.

Publications:

Rice, K. C. and Brossi, A.: Direct Conversion of S-(+)-Bulbocapnine into S-(+)-2-Hydroxy-10-Methoxyaporphine. Synthetic Communications, 8, 391-395 (1978).

Minamikawa, J. and Brossi, A.: Selective O-Demethylation of an Aromatic Methylether in the Presence of an Aromatic Methylemedioxy group. Tetrahedron Let. 3085-3086 (1978).

Minamikawa, J. and Brossi, A.: J. Can. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19216-03 LC

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Molecular Features of Colchicine Associated with Antimitotic and Antigout Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	H. G. Capraro	Visiting Fellow	NIAMDD-LC
	A. E. Jacobson	Research Chemist	NIAMDD-LC

COOPERATING UNITS (if any)

Istituto di Sanita, Rome (M. Iorio), Laboratory of Enviromental Biophys. (C. F. Chignell), School of Pharmacy, University of Mississippi, (Hufford); NCI-DR&D (M. Suffness); Heart and Lung Institute, NIH (J. V. Silverton).

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SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES x☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Optically pure (+)-colchicine binds substantially less to tubulin in vitro than the natural alkaloid. An efficient conversion of colchicine into demecolcine has been accomplished. Three phenolic colchicine analogs have been characterized by physical measurements. The X-ray structure of a colchiceine hydrate has been obtained, showing that it is the keto enol of colchicine. It has been further substantiated that the N-acetamido group of colchicine is not necessary for tubulin binding. Compounds showing good binding to tubulin have been evaluated toxicologically and promising, less toxic compounds, have been submitted to NCI for in vivo evaluation. Speciosine, a rare alkaloid from Colchicum autumnale, is substantially less toxic than colchicine.

A sample of optically pure (+)-colchicine, the optical isomer of natural colchicine, has now been prepared (M. Iorio) by an improved procedure, and found to bind substantially less to tubulin in vitro than the natural alkaloid.

A practical conversion of colchicine into the rare natural alkaloid demecolcine has been accomplished. Colchicine is converted into demecolcine in 5 steps, with 50-60% overall yield by the following sequence: Colchicine→ trimethylcolchicinic acid N-trifluoroacetyl-N-deacetylcolchiceine→N-trifluoroacetyl-N-deacetylcolchicine with separation from its iso-isomer→N-trifluoroacetyl-N-methyl-colchicine and hydrolysis. Several grams of chemically pure demecolcine have been prepared by this route, making demecolcine readily available for further chemical transformations. Hydrolysis of N-deacetyl-N-trifluoroacetyl colchicine affords N-deacetylcolchicine, a compound until now difficultly obtained.

Several colchicine analogs obtained in the course of these investigations have been evaluated with regard to tubulin binding in vitro, and those showing good binding capacity have been further evaluated toxicologically in mice after i.p. administration. The following compounds, showing good binding to tubulin and lesser toxicity than colchicine, have been submitted to the Cancer Institute for in vivo evaluation: N-formyldemecolcine, N-trifluoroacetyl-demecolcine, N-methyldemecolcine and speciosine. The latter compound, a rare alkaloid from Colchicum autumnale, binds well to tubulin but shows substantially lower toxicity than colchicine. Whether this is due to lower absorbtion will become evident from its in vivo evaluation, but with regard to its therapeutic index speciosine may well represent a much better tolerated colchicine derivative, with potential use in gout and antitumor therapy.

Several phenolic colchicines, including 1-O-demethylcolchicine, 2-O-demethylcolchicine and 3-O-demethylcolchicine have been characterized by physical techniques including ^{13}C- and ^{1}H-nmr data. All these phenolic compounds bind substantially less to tubulin than colchicine, but the binding is improved by O-acylation. The synthesis of phenolic demecolcines, starting from phenolic colchicines, is now being attempted, applying in part sequences utilized in the conversion of colchicine to demecolcine.

The finding that the N-acetamido groups are not necessary for interaction with tubulin has been substantiated with a deactemido-dehydrocolchicine preparation obtained by Hoffman degradation of a N-methyldemecolcine quaternary salt. This compound shows excellent binding to tubulin and this class of compound is being investigated vigorously.

The structure of colchiceine, obtained as a mono-hydrate, by X-ray crystalography, has shown that it is a trimer of the enol ketone corresponding to colchicine. The three colchiceine molecules are held together by intra- and intermolecular hydrogen bonding involving a water molecule.

The studies accomplished so far have revealed that the C-7 substituent in colchicine can be varied considerably, affording compounds with substantially lesser toxicity in mice after i.p. administration. Speciosine is thus

far our best compound, and its evaluation in vivo will largely influence our program, which will include phenolic demecolcines, N-deacetamidocolchicines and in addition allocolchicine. We are hopeful that this program will lead to a colchicine analog with a much better therapeutic index and hopefully with potential as an improved colchicine for therapeutic application in gout and as an antitumor agent.

Publications:

Iorio M., Silverton, J. V. and Brossi, A.: 7-Oxo-deacetamidocolchiceine and 7-Benzylimino-deacetamidocolchiceine: Two Novel Products from the Base Catalysed Reaction (-)-N-Benzylidene-deacetylcolchiceine [1]). Helv. Chim. Acta., 61, 1213-1220 (1978).

Capraro, H. G. and Brossi, A.: Simple Conversion of Colchicine into Demecolcine. Helv. Chem. Acta., 62, 965-970 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19217-02LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
3-Phenoxy substituted Phenethylamines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	H. G. Capraro	Research Fellow	NIAMDD-LC

COOPERATING UNITS (if any)
Hoechst-Roussel Co., Sommerville, New Jersey

LAB/BRANCH
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Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been discontinued.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19218-02 LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

N-Acetylated Pyrimethamine as Antimalarials

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief Medicinal Chemistry	NIAMDD-LC
OTHER:	N. Acton	Guest Worker	NIAMDD-LC

COOPERATING UNITS (if any)

Divison of Medicinal Chemistry, Walter Reed Army Institute of Research (Dr. T. Sweeney).

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NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

N-acetylated pyrimethamines do not show sustained release activity in the respective antimalarial screening. Several of the derivatives are however active antimalarials. Their toxicity in comparision with pyrimethamine is now being evaluated.

Four N-acetylated pyrimethamines have been prepared and characterized. Three of them had curative effects in malaria infected mice and are now being evaluated toxicologically in comparision with the parent drug. The possibility that N-acylated pyrimethamines are being formed during the incorporation of pyrimethamine into biopolymers of the polylactic type is being considered, and pyrimethamine lactates are being prepared.

Publications:

Acton, N. and Brossi, A.: Antimalarial Activity of N-Acetylated Pyrimethamines, J. Med. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19219-02LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Synthesis of 3-Deoxymorphine and Dihydro Congeners, and Their Pharmacological Evaluation.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	A. E. Jacobson	Research Chemist	NIAMDD-LC
	K. C. Rice	Research Chemist	NIAMDD-LC
	J. Reden	Guest Worker	NIAMDD-LC
	M. F. Reich	Staff Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

Laboratory of General and Comparative Biochemistry, NIMH, ADAMHA

LAB/BRANCH
Laboratory of Chemistry

SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We determined the effect of the removal of the phenolic hydroxyl group of opioids on antinociception, receptor binding affinity, and abuse potential. One of these compounds, 3,6-dideoxydihydromorphine was at least twice as potent as morphine, or dihydromorphine, as an antinociceptive. It appeared to have a dependence capacity very much like morphine in single-dose suppression studies in the rhesus monkey. Its binding affinity to the opiate receptor was about one-third that of morphine. Thus, the phenolic hydroxyl is not necessary for potent antinociceptive activity, nor for the development of physical dependence of the opiate-type. However, the phenolic hydroxyl does aid an opioid in binding to its receptor.

Catalytic reduction of the 3-tetrazolyl ether derivatives of dihydromorphine provided the entry into the 3-deoxydihydro series. The prototype, 3-deoxymorphine, was prepared by LAH reduction of 3-deoxy-N-carbethoxymorphinone, obtained *via* its 7-phenylseleno derivative. Thus, 3-deoxydihydromorphine, 3-deoxydihydronormorphine, 3-deoxymorphine, 3-deoxydihydromorphinone, 3-deoxy-N-allyldihydronormorphine, and 3,6-dideoxydihydromorphine were prepared.

It is probable that, by removing the phenolic hydroxyl group, we have increased the lipophilicity of these compounds, allowing more efficient transport to the brain. Although lessened receptor affinity was noted, the overall balance for, at least, the 3,6-dideoxydihydromorphine resulted in increased *in vivo* antinociceptive activity. The remaining oxygen atom in that molecule, in the epoxy ring, aided in providing the observed increased potency. An alternative explanation, metabolic transformation *in vivo* to an active metabolite causing the observed increase in potency, is being explored.

Publications:

1. Reden, J., Reich, M. F., Rice, K. C., Jacobson, A. E., Brossi, A., Streaty, R. A., and Klee, W. A.: Deoxymorphines: Role of the Phenolic Hydroxyl in Antinociception and Opiate Receptor Interactions. *J. Med. Chem.* *22*: 256-259, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19220-02LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Synthesis and Antinociceptive Activity of 7-Methoxycodeine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. E. Jacobson	Research Chemist	NIAMDD-LC
OTHER:	I. Iijima	Research Scientist	NIAMDD-LC
	J. Minamikawa	Research Fellow	NIAMDD-LC
	K. C. Rice	Research Chemist	NIAMDD-LC

COOPERATING UNITS (if any)

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SECTION
Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been completed.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19221-02 LC

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Dimers of Natural and (-)- and Synthetic (+)-Sinomenine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	J. Minamikawa	Research Fellow	NIAMDD-LC
	I. Iijima	Research Scientist	NIAMDD-LC

COOPERATING UNITS (if any)

NCI-DR&D (H. B. Wood, Jr.)

LAB/BRANCH Laboratory of Chemistry

SECTION Medicinal Chemistry

INSTITUTE AND LOCATION NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Work completed.

PHS-6040
(Rev. 10-76)

The dimers obtained from natural (-)- and synthetic (+)-sinomenine were fully characterized. The two isomers of each series can be converted into each other by heating. All dimers contain solvents of crystallization which can only be removed with disintegration of the crystal modification, making it impossible to determine their absolute structure by conventional X-ray crystallography.

Publications:

Minamikawa, J., Iijima, I. and Brossi, A.: Dimers of Natural (-)- and Unnatural (+)-Sinomenine. Heterocycles 10, 79 (1978).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19222-02 LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Condensation Products of Retinals with 1,3-Diketones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	N. Acton	Guest Worker	NIAMDD-LC

COOPERATING UNITS (if any)
Dr. M. Sporn, Lung Cancer Branch, Division of Cancer Cause and Prevention, NCI

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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A large series of condensation products of all trans retinal with available and newly prepared 1,3-diketones has been prepared. Relationship between biological activity, photostability and structure has been established. Chemical transformation in ring A of retinylidene dimedone, one of the more promising compounds prepared, has been executed. Several new derivatives of all-trans retinoic acid, the most active compound found thus far, have been prepared, including esters and amides.

About 30 condensation products of all-trans retinal with a variety of 1,3-diketones, including substituted cyclohexanediones obtained from Hoechst (Germany), 1,3-cycloheptanedione, 1,3-cyclooctanedione and 1,3-cyclononanedione have been prepared. Condensation of 13-cis retinal with dimedone afforded the all-trans condensation product due to isomerization of the aldehyde under the condensation conditions. Epoxidation of retinylidene dimedone with peracid afforded the crystalline epoxide which was converted into a dihydrofurane derivative under acidic conditions. All these compounds are presently being evaluated. It seems that a ring larger than a six membered ring at the terminal end group does not reduce biological activity, and it seems that some of the compounds have improved stability.

In order to find a new active modification of the terminal end group in all-trans retinoic acid, several novel amides and esters, such as the cholesteryl ester and the pivaloylester, were prepared. These investigations will be further guided by the outcome of their biological evaluation. Other isomers of retinal such as the 9-cis isomer will be included in these studies when available.

A study regarding photostability and stability in formulations will be initiated once the most promising candidate has been found.

Publication: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19223-02 LC

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Tetrahydropapaveroline

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	C.P. Mak	Visiting Fellow	NIAMDD-LC
	A.E. Jacobson	Research Chemist	NIAMDD-LC
	J. Reden	Guest Worker	NIAMDD-LC

COOPERATING UNITS (if any)

P. Skolnick, Lab of Bioorganic Chemistry, NIH; G. Cohen, Mount Sinai, New York

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several potential metabolites and transformation products of THP, a urinary excretion product in patients under L-dopa therapy have been prepared and are being evaluated. THP is unstable at pH above 7 and converted into dibenzopyrrocolines and dimers of this system. The first dibenzopyrrocoline dimer has been recognized and characterized.

Tetrahydropapaveroline (THP), the first Mammalian Alkaloid, isolated from the urine of Parkinsoinan patients under L-dopa therapy, is of considerable interest. It acts as a false transmitter of dopamine, but its origin and its significance are not clear. To get some further insight with regard to its biological significance, several compounds, chemically related to THP, and possibly formed as metabolites from THP or its precursors, were synthesized. This included the 6- and 3'-O-methylethers of THP, its 1-carboxylic acid precursor, obtained from dopamine by condensation with 3,4-dihydrophenyl pyruvic acid, and a tetrahydroxy-dibenzopyrrocoline in form of its tetra-acetate. The latter system is known to be obtained easily by oxidative coupling of THP. It has been shown in our laboratory that dibenzopyrrocoline is readily formed from THP in the presence of oxygen at pH 7 and above. All these THP related compounds are now being evaluated as false transmitters, in assays measuring catecholamine receptor interaction. THP and trimetoquinol, a commercially available bronchodilator, chemically related to THP, will be used as standards. It is questionable whether these studies will allow any predictions with regard to the origin of THP, but they certainly will reveal information whether THP is the biologically active compound or whether its demonstrated activities are related to metabolites or chemical transformation products. The oxidation of THP under physiological conditions is of interest since THP is chemically labile at pH 7 and above, suggesting that some reported data might be of questionable value. During the preparation of dibenzopyrrocolines it was found that a compound reported in the literature has the wrong structure and was indeed the first dimeric dibenzopyrrocoline prepared. Dimerization of dibenzopyrrocolines, rather than aromatization, occurs readily. The outcome of the biological evaluation of these compounds will determine the further research program.

Publications:

C. P. Mak and A. Brossi: Bis-(tetrahydroxy-dibenz[b,g]indolizine) by air oxidation of tetrahydropapaveroline, Tetrahedron Letters, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19224-01LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Microbiological oxidation of Morphines and Morphinans.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	A. E. Jacobson	Research Chemist	NIAMDD-LC
	F. Hsu	Staff Fellow	NIAMDD-LC

COOPERATING UNITS (if any)

Dr. D. Perlman, University of Wisconsin, School of Pharmacy, Madison

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NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A microbial system for the conversion of 3-deoxyopioids to their phenolic hydroxyl derivative is being sought. This conversion may provide a simpler route to the natural alkaloids, utilizing chemical and microbiological transformations.

PHS-6040
(Rev. 10-76)

Chemical procedures have provided a practical route to 3-deoxydihydro-morphine. Microbiological conversion of this type of compound would complete the overall transition from simple starting materials to morphine-like alkaloids.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19225-01LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Investigation of an unusual 7-ketodihydromorphinan

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	J. Minamikawa	Research Fellow	NIAMDD-LC
	K.C. Rice	Research Chemist	NIAMDD-LC
	A.E. Jacobson	Research Chemist	NIAMDD

COOPERATING UNITS (if any)

Dr. J. Silverton, Lab. of Chemistry, NHLI; Williams, Hoffman-LaRoche, Inc., N.J.

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Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

7-Keto-3-methoxy-4-hydroxy-5,6-dihydromorphinan was found to exist in an equilibrium mixture with its tautomer, 7-keto-6-deoxydihydrocodeine. An x-ray crystallographic study showed that the molecule was in a space group seen only twice before with organic compounds. It existed, in the solid state, in the form of a double helixan interlacing pair of helices joined by hydrogen bonds. Structures for the compounds involved in the tautomeric equilibrium were deduced from a carbon-13 NMR study, proton NMR, and other spectroscopic techniques.

PHS-6040
(Rev. 10-76)

One of the essential steps in our preparation of the unnatural enantiomers of biological important opioids was the cyclization of dihydrosinomenine to dihydrocodeinone. A by-product was consistently observed to occur during that reaction. Since the availability of natural sinomenine and, thus, its dihydro derivative, was extremely limited, we undertook the structural elucidation of this by-product with the hope that knowledge of its structure would enable us to suppress its formation. The by-product was separated from the reaction mixture and various spectroscopic measurements, including x-ray crystallography, proved the existence of a tautomeric pair of compounds and indicated their structures.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19226-01LC

PERIOD COVERED
October 1, 1979 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Synthesis and Pharmacology of Irreversible Inhibitors of the Benzodiazepine Receptor.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. C. Rice	Research Chemist	NIAMDD-LC
OTHER:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
	P. Skolnick	Pharmacologist	NIAMDD-LBC

COOPERATING UNITS (if any)

J. Tallman, NIMH-BP, S. Paul, NIMH-CP

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Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XXX☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several benzodiazepines containing reactive functional groups were synthesized as specific, irreversible inhibitors of the benzodiazepine receptor in order to further study and characterize these receptors, through which the pharmacological properties of benzodiazepines are expressed. One of these inhibitors, Irazepine, binds irreversibly and specifically to the benzodiazepine receptor and represents the first example of this type of inhibitor. Irazepine is a relatively stable, crystalline substance and is easily prepared from known compounds in one step.

The benzodiazepines are among the most widely prescribed drugs worldwide, and have recently been shown to exert their pharmacological actions by reversible interaction with high affinity binding sites in the mammalian central nervous system. In order to further characterize these receptors and to more clearly define the mechanism of action of these drugs, we have designed and synthesized benzodiazepines which act as specific, irreversible inhibitors of the receptors. These inhibitors function by specific interaction with the receptor, followed by covalent bond formation between a reactive functional group in the benzodiazepine and suitable functionality in the receptor.

Receptors irreversibly labeled (identified) in this manner are differentiated from other proteins present in brain tissue and are thus much more amenable to study than the unlabeled counterparts. Irazepine, a benzodiazepine containing the reactive isothiocyanate moiety, was recently synthesized and proved to be the first example of a specific irreversible inhibitor of benzodiazepine receptor. Very recent work with a second generation inhibitor suggests the existence of two types of benzodiazepine receptors which may function separately to regulate the different central effects of benzodiazepines and further work along these lines is in progress. It seems likely that Irazepine and similar irreversible inhibitors will be of great value in isolation, purification and characterization of the benzodiazepine receptors as well as in estimation of receptor turnover in vivo.

Publication:

Rice, K. C., Brossi, A., Tallman, J., Paul, S., Skolnick, P.: Irazepine, a noncompetative irreversible inhibitor of ^{3}H-diazepam binding to benzodiazepine receptors. Nature, 278, 854-855 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19227-01LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Synthesis of Racemic and Optically Active Pavinan and Isopavinan Alkaloids from N-Norreticulines.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	Chief, Medicinal Chemistry	NIAMDD-LC
OTHER:	K.C. Rice	Research Chemist	NIAMDD-LC
	J. Reden	Visiting Scientist	NIAMDD-LC
	W. Ripka	Guest Worker	NIAMDD-LC

COOPERATING UNITS (if any)

None

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Medicinal Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The racemic and natural forms of a number of pavinan and isopavinan alkaloids were prepared in several steps from 4-methoxy-N-acyl-N-norreticulines which in turn, are easily obtained by DDQ oxidation of the corresponding N-acyl-N-norreticulines in methanol. Treatment of (+)-N-formyl-N-northalidine and N-formyl-N-norbisnorargemonine with hydrazine afforded the corresponding N-nor bases which were O-demethylated to the tetrols. Pharmacological investigation of a number of compounds prepared in this study is in progress.

Oxidation of racemic and optically active N-norreticulines with DDQ in methanol at -78°C afforded the corresponding 4-methoxy derivatives that were readily transformed into a variety of racemic and optically active pavinans and isopavinans. Either the pavinan or the isopavinan carbon skeleton could be obtained from the same 4-methoxy intermediate. Treatment of (±)-4-methoxy-N-formyl-N-norreticuline with 1% methanesulfonic acid in acetonitrile readily afforded the isopavinan (±)-N-formyl-N-northalidine. Reduction of the N-formyl group with borane afforded (±)-thalidine and cleavage of this functionality with hydrazine afforded (±)-northalidine. Heating 4-methoxy-N-formyl-N-norreticuline at 200°C for 1 hr gave the corresponding 3,4-dehydro compound which regioselectively cyclized under the same acid conditions described above to N-formyl-N-norbisnorargemonine. Reduction of this pavinan with borane afforded (±)-bisnorargemonine and hydrazinolysis afforded the corresponding N-nor compound. The N-norpavinan and N-norisopavinan were O-demethylated with 48% HBr to the tetrahydroxy compounds which could, in principle, be formed by oxidation of tetrahydropapaveroline to the 4-hydroxy derivative and cyclization. In the optically active series, (-)-N-norreticuline was converted t the (+)-N-carbethoxy derivative which was transformed to the corresponding N-carbethoxy-N-norpavinan or N-norisopavinan using a reaction sequence similar to that employed in the N-formyl series. Reduction of the N-carbethoxy derivatives with LAH afforded the optically pure natural alkaloids (-)-thalidine and (-)-bisnorargemonine. Various aspects of the pharmacology of these compounds is under investigation.

Publications:

Rice, K.C. and Brossi A.: Expedient Synthesis of Racemic and Optically Active N-Norreticulines 6'-Bromo and N-Substituted Congeners. J. Org. Chem., in press.

Rice, K.C., Ripka, W.C., Reden, J. and Brossi, A.: Pavinan and Isopavinan. Synthesis of Racemic and Natural Thalidine, Bisnorargemonine and Congeners from N-Norreticuline.[1a] J. Org. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19228-01LC

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Conversion of dihydromorphine to 4-hydroxy-6-ketomorphinan and 3-deoxydihydromorphine.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Brossi	**Chief, Medicinal Chemistry**	NIAMDD-LC
OTHER:	F. Hsu	Staff Fellow	NIAMDD-LC
	A. E. Jacobson	Research Chemist	NIAMDD-LC
	K. C. Rice	Research Chemist	NIAMDD-LC

COOPERATING UNITS (if any)

None

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Laboratory of Chemistry

SECTION

Medicinal Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chemical entry into the 6-ketomorphinan series has been successfully accomplished. The 4-hydroxy-6-ketomorphinan, which has been, hitherto, difficulty accessible can be converted to 3-deoxydihydromorphine, a possible substrate for microbiological oxidation.

The opiate alkaloids (morphine or dihydromorphine), through catalytic reduction of their tetrazolyl ether, were converted to 3-deoxyopioids. Oppenauer oxidation of 3-deoxydihyromorphine followed by aluminium amalgam reduction gave 4-hydroxy-6-ketomorphinan, which was easily reconvertible to 3-deoxydihydromorphine. Reduction of the 6-ketomorphinan provided the epimeric 4,6-α and β-dihydroxymorphinan. The ketomorphinan was, essentially, morphine-like in antinociceptive activity; the epimeric 6-hydroxy compounds had only weak antinociceptive activity.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19401-14 LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Synthetic Photochemistry and Photobiology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bernhard Witkop Chief, LC NIAMDD

COOPERATING UNITS (if any) O. Yonemitsu, Y. Kanaoka and T. Iwakuma, Univ. of Hokkaido, Sapporo, Japan; Masako Nakagawa, Faculty of Pharmaceutical Science, Chiba Univ. Japan; I. L. Karle, U. S. Naval Research Lab., Wash. D. C., Prof. L. Jaenecke, Univ. Cologne, Cologne, Germany

LAB/BRANCH
Laboratory of Chemistry

SECTION
Metabolites

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. The synthesis of novel heterocycles of metabolic significance and with potential for antiviral and CNS activity has been accomplished by photocyclizations and dimerizations.

2. Novel tryptophan metabolites were synthesized and their relative and absolute stereochemistry and chirality established by Roentgen Ray Analysis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Reactions for the selective modification of simple amino acids in proteins and of purines adn pyrimidines in nucleic acids are needed for the study of the composition and function of their active sites. Although extensive interest to develop such reactions has been shown by many investigators, photochemical methods have been elaborated with a number of homo- and heterocyclic models which make it possible to deliver selectively a distinct amount of energy to a system. The photochemical behavior of free and bound aromatic amino acids and the corresponding pharmacodynamic amines have been investigated. Studies continue with new heterocyclic systems.

Methods Employed: Ultraviolet irradiation, flash photolysis thin-layer chromatography, silica gel and ion exchange column chromatography, paper chromatography and electrophoresis, UV, IR, NMR and mass spectroscopy.

Major Findings: A novel bacterial hydroxylase introduces a β-hydroxyl group into the side chain of certain tryptophan derivatives (Hayaishi). The absolute stereochemistry of synthetic erythro- and threo-hydroxy-tryptophans was established by x-ray crystallography.

Another α-hydroxy derivative, i.e., 5-hydroxyindole-3-lactic acid serves as the precursor of a sex-determining hormone in blepharisma Japonicum whose chemistry and biosynthesis is being studied.

Significance to Biomedical Research and the Program of the Institute: New approaches to novel heterocyclic systems of potential CNS activity.

Proposed Course: Extension into homo- and heterocyclic systems relating to pharmacodynamic amines and to potential antiviral cage compounds.

Honors: Ferrosan Jubilee Lecturer
University of Copenhagen
April 1979

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19402-06

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Interferon Induction and Action. The Antiviral Activity of Nucleoside Analogs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. F. Torrence	Research Chemist	NIAMDD, LC
OTHER:	B. Witkop	Chief, Lab. of Chem.	NIAMDD, LC
	M. I. Johnston	Staff Fellow	NIAMDD, LC
	J. Iami	Visiting Fellow	NIAMDD, LC

COOPERATING UNITS (if any) Erik De Clercq, Rega Institute, University of Leuven, Belgium; Mathias P. Mertes, University of Kansas, Lawrence, Kansas; D. Epstein, NIAMDD, LEP; R. M. Friedman, NIAMDD, LEP; K. Zoon, NIAMDD, LCB

LAB/BRANCH

Laboratory of Chemistry

SECTION

Metabolites

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.8	

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Interferon-induced enzyme activities, such as the oligo(2'→5')adenylate synthetase and the 67K protein kinase, are investigated with a goal of understanding their role in the action of interferon, the induction of interferon by double-stranded RNA (dsRNA) and, perhaps, control of cell-growth and differentiation. A new assay for the oligo(2'→5')adenylate synthetase has been developed and this has permitted the beginning of a study of the ability of differently modified dsRNA's and nucleic acids to activate this enzyme from mouse L cells. A similar study is being conducted with 67K protein kinase activity from extracts of mouse L cells. Evidence has been gathered that suggests the importance of a cellular phosphatase activity in determining the net labeling of the 67K protein by γ-^{32}P-ATP.

Project Description:

Objectives: The structural, conformational and biochemical bases that account for the observed stringent structural requirements for nucleic acid interferon induction is sought. Interferon-induced enzymes, especially those apparently dependent on nucleic acids for activation, are studied in order to understand them as nucleic acid receptor systems as well as to elucidate their fundamental cellular role. Particular emphasis is currently placed on understanding the phenomenon of the dsRNA-mediated inhibition of protein synthesis in interferon-treated cells.

The antiviral and antimetabolic properties of new nucleoside analogs is also under investigation, mostly on a collaborative basis.

Methods Employed: Thin-layer, paper, ion-exchange, gel and other types of column chromatography, liquid scintillation counting, ultracentrifugation, ultraviolet, visible, infrared and nuclear magnetic resonance, spectroscopy, mass spectroscopy, electron spin resonance, circular dichroism, tissue culture, gel electrophoresis.

Major Findings:

(1) Interferon, dsRNA and the oligo(2'→5')adenylate synthetase (Johnston, Friedman and Torrence).

To date, 2',5'-oligoadenylate synthetase has been assayed by two different methods: i) in solution; ii) bound to dsRNA attached to an insoluble support. Although the latter method offers some degree of enzyme purification, it is extremely cumbersome if different nucleic acids are to be evaluated as enzyme activators. The former method may not always permit synthesis of maximal concentrations of active 2',5'-oligoadenylates, since some cell extracts may contain high levels of inactivating enzymes.

We report here a new assay method based on adsorption of the oligoadenylate synthetase to 2',5'-ADP-Sepharose at 4°; incubation at 30° in the presence of ATP and dsRNA produced a heat stable protein synthesis inhibitor. This inhibitor was formed in concentrations similar to those obtained using the $(I)_n.(C)_n$-Sepharose method under identical conditions. Radiolabeled inhibitor, prepared from [^{3}H]-ATP, was characterized as 2',5'-ppp$(Ap)_n$A by chromatography on a Tener column and by thin-layer chromatography, before and after digestion with bacterial alkaline phosphatase. Under our experimental conditions at 30°, the bound murine enzyme was relatively stable over a period of three days. Inhibitor synthesis was linear for a least 5 hr, and was dependent on both $(I)_n.(C)_n$ and ATP concentrations.

This assay has been used to investigate the ability of various nucleic acids to activate the 2',5'-oligoadenylate synthetase of mouse L cells. For instance, 2'-O-methylation of $(I)_n.(C)_n$ or $(A)_n.(U)_n$ gave polymers that did not activate the enzyme. The dsRNA, $(I)_n.(Br^5C)_n$, which was completely inactive as an inhibitor of protein synthesis, was a potent activator of the synthetase under our conditions.

(2) Interferon, dsRNA and protein kinase (Epstein, Torrence and Friedman).

Under conditions approximating those of in vitro protein synthesis, phosphorylation of the 67K dalton protein is enhanced, in general, in the presence of those nucleic acids which inhibit protein synthesis in the L-cell cell-free system (J. Biol. Chem., 254: 1259-1267, 1979). For example, $(I)_n.(C)_n$ and $(A)_n.(U)_n$ stimulate incorporaton of label from γ-^{32}P-ATP into the 67K dalton protein while $(I)_n.(br^5C)_n$, which is inactive as an inhibitor or protein synthesis, has little effect. However, we have found conditions under which $(I)_n(br^5C)_n$ is approximately as effective as $(I)_n.(C)$ in stimulating incorporation of label from γ-^{32}P-ATP into 67K dalton protein. Upon transfer of the labeled 67K dalton protein from these latter conditions to those compatible with protein synthesis, there is a time-dependent decrease in label present in 67K dalton protein. This decrease is more rapid in the presence of $(I)_n.(br^5C)_n$ than in the presence of $(I)_n.(C)_n$.

These results suggest that dephosphorylation as well as phosphorylation determines the net incorporation of ^{32}P-label into the 67K dalton protein. Moreover, these results suggest the existence of a phosphatase activity which shows stronger apparent inhibition by $(I)_n.(C)_n$ than by $(I)_n.(br^5C)_n$.

(3) 5-Cyano-2'-deoxyuridine (Mertes and Torrence).

Earlier we found that 5-cyano-2'-deoxyuridine possessed significant anti-vaccinia virus activity on tissue culture. Moreover, we observed that this 5-cyanopyrimidine nucleoside inhibited the incorporation of labeled 2'-deoxyuridine into DNA, but had no effect on labeled deoxythymidine incorporation. On this basis, we suggested that 5-cyano-2'-deoxyuridine may be a potent inhibitor of thymidylate synthetase.

In order to test this hypothesis, we prepared the 5'-monophosphate of 5-cyano-2'-deoxyuridine and evaluated it as an inhibitor of thymidylate synthetase from Lactobacillus caesi. 5-Cyano-2'-deoxyuridine 5'-monophosphate was a potent competitive inhibitor with a K_i of 5.5×10^{-7} M. Irreversible enzyme inhibition by this compound could not be detected.

Significance to Biomedical Research and the Program of the Institute: Studies such as those described herein showed lead to a better understanding of the mechanism of interferon action. They may also help unravel certain cellular control mechanisms that may be important not only as an antiviral defense but perhaps also in the control of growth, differentiation and development and thus perhaps neoplasia.

Publications:

Torrence, P. F. and Witkop, B. "The Synthesis of Poly(2'-azido-2'-deoxyuridylic acid)" in Nucleic Acid Chemistry, Part 2, Eds. L. B. Townsend and R. S. Tippan, p. 977-988 (1978).

Torrence, P. F., Waters, J. A. and Witkop, B. "2'-Deoxy-5-(thiocyanato) uridine [1-(2-Deoxy-B-D-Erythro-pentofuranosyl)-5-(thiocyanato)-uracil]" in Nucleic Acid Chemistry, part 1, eds L. B. Townsend and R. S. Tipsan, p. 367-370 (1978).

De Clercq, E., Torrence, P. F., Stollar, B. D., Hobbs, J., Fukui, T., Kakiuchi, N. and Ikehara, M. "Interferon Induction by a 2'-modified Double-Helical RNA, Poly(2'-azido-2'-deoxyinosinic acid).Polycytidylic acid", Euro. J. Biochem. 88, 341-349 (1978).

De Clercq, E. and Torrence, P. F. "Nucleoside Analogs with Selective Antiviral Activity", J. Carbohydrates.Nucleosides.Nucleotides" 5, 187-224 (1978).

Huang, G.-F. and Torrence, P. F. "Nitration of Cytosine, 1-Methylcytosine and 2'-Deoxycytidine 5'-Monophosphate by Nitronium Tetrafluoroborate in Sulfolane. Preparation of 5-Nitro-2'-Deoxycytidine". J. Carbohydrates.Nucleosides. Nucleotides 5, 317-327 (1978).

Mertes, M. P., Chang, T.-C., DeClercq, E., Huang, G.-F. and Torrence, P. F. "5-Nitro-2'-deoxyuridine is a Potent Irreversible Inhibitor of Lactobacillus caesi Thymidylate Synthetase", Biochem. Biophys. Res. Comm. 84, 1054-1059 (1978).

Torrence, P. F. and Friedman, R. M. "Are Double-stranded RNA-Directed Inhibition of Protein Synthesis in Interferon-treated Cells and Interferon Induction Related Phenomena? J. Biol. Chem. 254, 1259-1267 (1979).

Torrence, P. F., Huang, G.-F., Edwards, M. W., Bhooshan, B., Descamps, J. and De Clercq, E. "5-Substituted Uracil Arabinonucleosides as Potential Antiviral Agents". J. Med. Chem. 22, 316-319 (1979).

Torrence, P. F., Friedman, R. M., Kirk, K. L., Cohen, L. A. and Creveling, C.R. "2-Fluorohistidine: Effects on Protein Synthesis in Cell-Free Systems and in Mouse L Cells". Biochem. Pharmacol., 28, 1565-1567 (1979).

Torrence, P. F., De Clercq, E., Descamps, J., Huang, G.-F., and Witkop, B. "C-5 Substituted Pyrimidine Nucleosides as Antiviral Agents". Frontiers in Bioorganic Chemistry, Pergamon Press, Ltd., in Press.

De Clercq, E., Descamps, J., Barr, P. J., Jones, A. S., Serafinowski, P., Walker, R. T., Huang, G.-F., Torrence, P. F., Schmidt, C. L., Mertes, M. P., Kulikowski, T. and Shugar, D. "Comparative Study on the Potency and Selectivity of Anti-Herpes Compounds" in Antimetabolites in Biochemistry, Biology and Medicine, Eds. J. Skoda and P. Langen, Pergamon Press, Oxford, 1979, p. 275-285.

Chang, T.-C., Edwards, M. W., Torrence, P. F. and Mertes, M. P. "5-Cyano-2'-deoxyuridine-5'-phosphate: A Potent Competitive Inhibitor of Thymidylate Synthetase. J. Med. Chem., in Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19408-06 LC

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Central Nervous System Agents Based on the Piperidine and Pyrrole Ring Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James A. Waters	Research Chemist	LC NIAMDD
OTHER:	A. E. Jacobson	Research Chemist	LC NIAMDD
	E. L. May	Adjunct Professor	U. of Virginia
	J. W. Daly	Laboratory Chief	LBC NIAMDD
	P. Skolnick	Research Chemist	LBC NIAMDD
	E. Brochmann-Hanssen	Professor	U. Calif.
	S. S. Parmer	Professor	U. N. Dakota

COOPERATING UNITS (if any)

Antiepileptic Drug Development Program, Epilepsy Branch, NINCDS, NIH

LAB/BRANCH

~~Laboratory of Chemistry~~

SECTION

Metabolites

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Aromatic and heterocyclic esters of the non-quaternary carbon-4-piperidinol system exhibited analgesic activity in mice, no physical dependence liability of the morphine-type in monkeys and rats and little or no binding to the opiate receptor. A 3-substituted piperidine of this series showed narcotic antagonist properties similar to that displayed by Cyclazocine. Piperidinol esters, dissimilar to those displaying good analgesic activity, were active in the maximal electroshock antiepileptic assay in mice. Pyrrole-carboxylic acids showed good activity in antiinflammatory and antiproteolytic assays. Certain substrates within these three disciplines are being studied for their respective receptor interaction.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To design, synthesize and assay non-quaternary piperidinol esters, aralkylpiperidines and pyrrolecarboxylic acids as potential non-opiate analgesics, narcotic antagonists, antiepileptics and antiinflammatory agents and to study their receptor interactions.

Methods Employed: Numerous synthetic procedures have been used in the preparation of piperidinol esters, aralkyl piperidines, piperidine ethers and pyrrolecaroboxylic acids. Electronic, steric and lipophilic parameters were modified for their effect on transport, metabolism and receptor interaction. Pharmacological and receptor assays were carried out using established procedures.

Major Findings:

(1) Non-quaternary piperidinol esters exhibited analgesic potency in the codeine-morphine range in mice, no physical dependence liability of the morphine-type in monkeys and rats, and marginal or no binding to the opiate receptor in rat brain homogenates.

(2) 1-Methyl-3-carboethoxy-4-piperidinol 3,4-dimethoxybenzoate (cis isomer) is a narcotic antagonist with properties similar to that of Cyclazocine and longer acting than Nalorphine.

(3) Antiepileptic activity was displayed by certain aromatic piperidinol and quinuclidinol esters. Activity was in diphenylhydantoin-methsuximide range by the maximal electroshock assay.

(4) Pyrrole-2-carboxylic acid and other substituted acids exhibited activity in the antiinflammatory rat paw edema, heat-induced hemolysis and antiproteolytic assays.

Significance to Biomedical Research and the Program of the Institute: Analgesics, which lack the numerous side-effects of morphine and other opiates, especially physical dependence and abuse liabilities, narcotic antagonists are needed for clinical use in relief of pain and narcotic addiction. Anti-epileptic agents with a low incidence of toxic effects are needed for the control of the various epileptic seizures. Antiinflammatory analgesics of the non-steroidal type are useful in the treatment of inflammation and pain associated with arthritis.

Proposed Course: Radioactive and non-radioactive substrates are being used to study the following receptor interactions: (1) the non-opiate analgesic receptor; (2) the benzodiazepine receptor (antiepileptic); (3) prostaglandin synthetase inhibition (antiinflammatory).

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19409-04 LC

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Synthesis of Nucleosides with Possible Antiviral Properties

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Thomas F. Spande	Research Chemist	NIAMDD LC
OTHER:	Paul F. Torrence	Research Chemist	NIAMDD LC
	Jiro Imai	Research Chemist	NIAMDD LC

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Metabolites

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0		

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is temporarily inactive.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19410-01 LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Synthesis of Indolizidine Alkaloids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Thomas F. Spande — Research Chemist — NIAMDD LC

COOPERATING UNITS (if any)
John W. Daly, Chief, Lab. of Bioorganic Chemistry, NIAMDD

LAB/BRANCH
Laboratory of Chemistry

SECTION
Metabolites

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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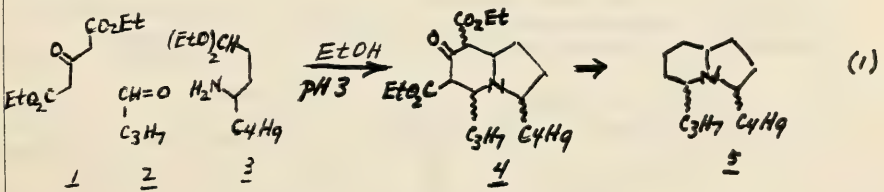
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Lions-Williamson Synthesis will be applied to the preparation of a 3,5-dialkyl indolizidine (eq. 1).



1 2 3 4 5 (1)

Compound 5 has been isolated (J. Daly) from the skin of the frog Dendrobates histrionicus. Insufficient material is available from that source for a thorough structure proof and for biological testing, necessitating a synthesis.

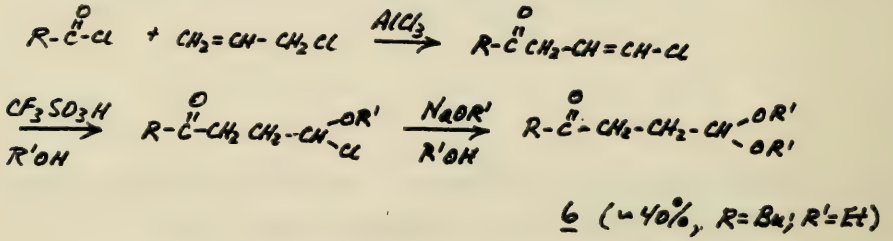
PHS-6040
(Rev. 10-76)

Project Description:

Objectives: A practical synthesis of 5 and a separation of the resulting mixture of 4-diastereoisomeric racemates should permit identification of the natural material as one of the diastereoisomers.

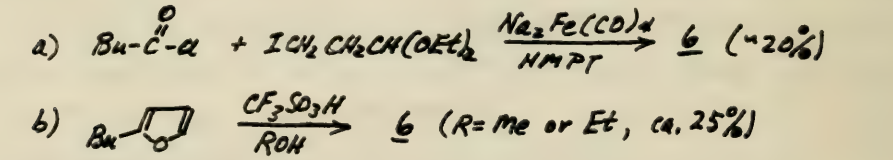
Methods Employed: IR, PMR, ^{13}CMR, mass spectrometry and column or HPLC techniques.

Major Findings: A practical new route to 3-oxoacetals 6 has been devised as follows:

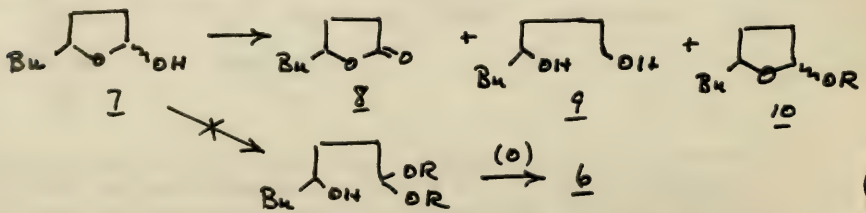


6 (~40%, R=Bu; R'=Et)

Such compounds may be useful in the synthesis of 2-alkyl furans. Compound 6 (R=Bu, R'=Et) can be converted in 2-steps to the key amino acetal intermediate 3. Two other routes (a,b) to 6 were developed but are less satisfactory.



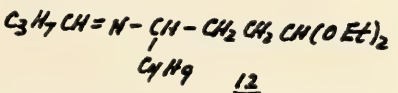
An attractive route using the lactol 7 obtained by partial reduction of commercial 3-hydroxyoctanoic acid lactone (8), gave in attempted acetalization, mixtures of lactone, diol and cyclic acetal 10. None or the



ring-opened hydroxy acetal 11 could be obtained. 8 and 9 Evidently arise from acid-catalyzed disproportionation of 7, even with Lewis-acid catalysis.

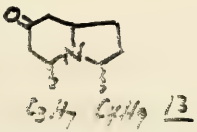
Significance to Biomedical Research and the Program of the Institute: A practical synthesis of 5 and other 3,5-dialkyl indolizidines should make supplies available for neuropharmacological testing.

Proposed Course: A modification of the classical 3-component L-W synthesis will be tried whereby a Schiff base intermediate 12 is performed, then allowed to react with 1 under the usual conditions of the reaction. This may improve



12

the yield and reduce by-products. Modified saponification and decarboxylation conditions for 4 will be tried as well as new reduction procedures for the resulting ketoamine 13. Manipulations on 13 may make possible stereoselective



13

formation of certain stereoisomers of 5.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19600-14 LC

PERIOD COVERED
October 1, 1978, through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Oxidation Mechanisms in Metabolic Processes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Louis A. Cohen Chief, Section on Biochemical Mechanisms LC NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemistry

SECTION
Biochemical Mechanisms

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been temporarily discontinued.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19601-12 LC

PERIOD COVERED
October 1, 1978, through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Chemical Modification and Cleavage of Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Louis A Cohen Chief, Section on Biochemical Mechanisms LC NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Biochemical Mechanisms

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been temporarily discontinued.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19602-13 LC

PERIOD COVERED

October 1, 1978, through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cleavage of Peptide Bonds by Intramolecular Participation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Louis A. Cohen Chief, Section on Biochemical Mechanisms LC NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been temporarily discontinued.

6-C-604C
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 19603-03 LC

PERIOD COVERED

October 1, 1978, through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Fluoro Analogs of Enzyme Substrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Louis A. Cohen	Chief, Section on Biochemical Mechanisms	LC NIAMDD
OTHER:	Kenneth L. Kirk	Research Chemist	LC NIAMDD
	Dwight Davis	Staff Fellow (Term. March 1979)	LC NIAMDD
	Olaronge Olubajo	Visiting Fellow (Arr. March 1979)	LC NIAMDD

COOPERATING UNITS (if any)

P. F. Torrence, LC NIAMDD
C. R. Creveling, LC NIAMDD
C. B. Klee, NCI

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Ring-fluorinated imidazoles show widespread and significant activity as enzyme substrates. 2-Fluorohistidine, but not the 4-fluoro isomer, is rapidly incorporated into newly synthesized animal or bacterial protein, in place of histidine. The resulting enzymes may be structurally intact, but some cannot function catalytically because the pK of the imidazole ring has been greatly depressed. 2-Fluorohistidine also serves to produce leucopenia, temporary regression of the mouse spleen, thymus, lymph nodes, and hypocellularity of the bone marrow. In mice infected with leukemia virus P-388, this analog prevents the normally massive increase in leukocyte levels and increases survival time up to 50%. Ultimate death may be due to causes other than leukemia. This antileukemic action may be unrelated to production of false protein, and may result from a block in the utilization of histidine as a one-carbon donor in purine biosynthesis. The block would result from the powerful inhibition of urocanase by 2-fluorourocanic acid, a metabolite of 2-fluorohistidine.

PHS-6040
(Rev. 10-76)

Project Description:

Investigation of the biochemical and pharmacological properties of ring-fluorinated imidazoles, as tools for the study of enzyme structure and function, and as potential therapeutic agents.

Objectives: Fluoro analogs of steroids, purines, pyrimidines and amino acids have been synthesized and tested, several having been found valuable in arthritis and cancer chemotherapy. A larger number have not been prepared because of the limited routes available for the introduction of fluorine. Despite the ubiquitous role of imidazoles in biological systems (RNA, DNA, histidine, histamine, etc.), no fluorinated imidazole had yet been prepared. The initial purpose of this work was to develop a general method suitable for the preparation of fluoroimidazoles, to prepare a series of such compounds and to test their abilities to serve as enzyme substrates or inhibitors and as replacement for histidine in proteins or for purines in DNA and RNA. To evaluate the fluoroimidazoles as antileukemic, antiviral, and immunosuppressive agents.

Methods Employed: Various enzyme assays, enzyme fractionation and purification methods are employed. Automatic cell counters to determine leukocyte levels. Standard techniques for manipulation of laboratory mice. Culture of yeasts and E. Coli.

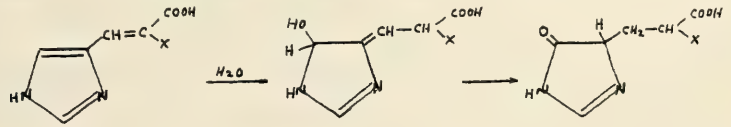
Major Findings: Studies are continuing on the incorporation of 2-F-His into various protein-synthesizing systems, from microorganisms to the whole mouse. Earlier experiments had involved 2-F-His with tritium labeling in the imidazole ring. The stability of the label to exchange is not quite as high as desirable, and efforts are in progress to prepare the same compound with a radioactive label on the amino acid side chain. For this purpose, the corresponding malonic acid derivative has been synthesized; decarboxylation in tritiated water should provide the desired label.

In earlier studies, we had found 4-F-His to be a modest substrate for histidine ammonia-lyase, but were somewhat surprised to discover that the deuterium isotope effect for the breaking of the β-CH bond is greater than that for histidine itself. Since the breaking of the β-CH bond must be involved in the rate-determining step for loss of ammonia from the amino acid, the result suggests that, contrary to expectation, fluorine decreases the acidity of the neighboring CH bond. Other investigators have shown that an ortho-fluorine atom reduces the rate of isotope exchange of the methyl group in toluene. Although the latter result is consistent with our enzyme data, it is also conceivable that 4-F-His binds to the enzyme in an anomalous conformation. We have now investigated 4-nitro-L-histidine as a substrate, in which compound the nitro group can be shown to have a considerable acidifying effect on the β-CH bond. Indeed, this compound shows no isotope effect whatever in the enzymatic abstraction of β-hydrogen, supporting our assumption that the ease of abstraction of the β-hydrogen is governed largely by electronic

effects. Furthermore, the rate of formation of 4-nitrourocanic acid is no longer dependent on the concentration of added metal ion, implying that the acidity of the β-hydrogen is now so great that its abstraction now precedes the rate-limiting loss of ammonia.

We had previously suggested that the antileukemic effect of 2-F-His may be related to the powerful inhibitory effect of its metabolite, 2-F-urocanic acid on urocanase. The important consequence of such inhibition would be a temporary interruption in the utilization of histidine as a one-carbon source for purine biosynthesis. Further examination of this postulate required the availability of sizable quantities of 2-F-urocanic acid, much more than could be prepared enzymatically. A chemical synthesis has now been developed, involving aryldiazonium coupling at C-2 of urocanic acid, stannous chloride reduction of the azo compound to 2-aminourocanic acid, and photochemical conversion of the latter compound to 2-fluorourocanic acid. Conjugates of this compound, which may be able to penetrate cell membranes, are being explored.

During the enzymatic conversion of urocanic acid (4, X = H) to imidazolonepropionic acid (6), the vinyl proton α to the carboxyl group is converted transiently to an allylic proton (5, X = H). Since allylic halides, in contrast to vinyl halides, are very susceptible to nucleophilic attack, it seemed reasonable to prepare 4 (X = halogen) as potential suicide substrates for urocanase. This compound has now been prepared with X = fluorine via a Wittig condensation. The analog is neither a substrate nor an inhibitor. and failed to retard the production of urocanic acid by histidine ammonia-lyase. Since the high electronegativity of fluorine may preclude protonation of the double bond (possibly the first step in the enzymatic protonation), studies are in progress with the less electronegative chloro and bromo analogs.



4 5 6

By use of our photochemical method for the introduction of aromatic fluorine, a convenient and direct route to optically active analogs of aromatic amino acids has been devised. In this way, 3-fluoro-L-tyrosine, 3,5-difluoro-L-tyrosine, and 5-fluoro-L-DOPA have now been prepared. [K. L. Kirk].

Significance to Bio-medical Research and the Program of the Institute: Studies to date indicate fluoroimidazoles to have a variety of properties in biological systems: enzyme inhibitors, histamine agonists, analogs of histidine, antibacterial, antiviral, and antileukemic agents. These compounds have already proved their usefulness in studies of enzyme structure

and function, as inhibitors of purine biosynthesis, involvement in cell division, etc. The possibility of therapeutic use for one or more of these compounds should also be considered.

Proposed Course: Additional fluoro analogs are being synthesized for biological evaluation. More detailed studies with enzymes are in progress, as well as efforts to isolate fluorine-containing proteins. The mechanism of the antileukemic effect is being investigated in detail.

Publications:

J. C. Reepmeyer, K. L. Kirk, and L. A. Cohen: 5-Fluoro-1-β-D-ribofuranosylimidazole-4-carboxamide. Sythesis of Fluoroimidazole Nucleosides via the Stannic Chloride Coupling Method, in "Nucleic Acid Chemistry," Part One, L. B. Townsend and R. S. Tipson, Eds., Wiley, 1978, pp 217-221.

P. F. Torrence, R. M. Friedman, K. L. Kirk, L. A. Cohen, and C. R. Creveling: 2-Fluorohistidine: Effects on Protein Synthesis in Cell-Free Systems in Mouse-L-Cells. Biochem. Pharmacol. (in press).

C. B. Klee, K. L. Kirk, and L. A. Cohen: 4-Nitro-L-histidine as a Substrate for Histidine-Ammonia Lyase: The Role of β-Hydrogen Acidity in the Rate-Limiting Step. Boichem. Biophys. Res. Commun., 87: 343-348, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19604-09 LC

PERIOD COVERED

October 1, 1978, through September 30, 1979

TITLE OF PROJECT (80 characters or less)

General Principles of Enzyme Catalysis and Simulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Louis A. Cohen	Chief, Section on Biochemical Mechanisms	LC NIAMDD
OTHER:	Michael King	Guest Worker	GWU
	Michael Channing	Staff Fellow (Arr. Apr. 1979)	LC NIAMDD

COOPERATING UNITS (if any)

None

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SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have advanced the theory that a major portion of enzymatic catalysis is achieved by activation of the substrate during binding. To support the theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen in a single, favorable conformation and by having the interacting functions brought into the closest possible juxtaposition. (stereopopulation control). These compounds undergo intramolecular reactions at rates comparable to those catalyzed by enzymes, sometimes even too fast to measure. It has been possible to generate high energy bonds within these models without energy input, simply using the free energy of formation of water and conformational restriction as the driving forces. In current work, models for sulfhydryl enzymes, oxidative phosphorylation and ion pumps are being developed. Techniques are being developed to elucidate molecular conformation in solution, based on ^{13}C and ^{19}F nmr spectroscopy, as well as computer calculations of conformational energies (CAMSEQ).

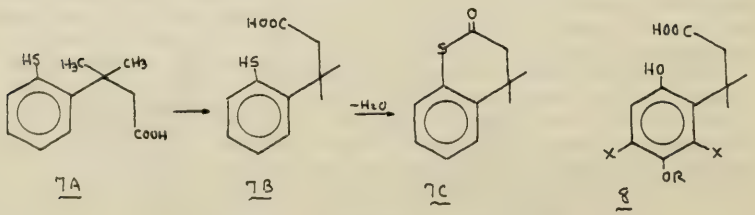
Project Description: Project No. Z01 AM 19604-09 LC

A study of the principles used by enzymes to accelerate chemical reactions. Model compounds are designed and synthesized which are capable of undergoing various reactions at rates comparable to those of enzymes, and under the same reaction conditions. Recognition of the principal devices used by enzymes will permit the design of more effective drugs and enzyme inhibitors or substrates.

Objectives: The rates of enzyme-catalyzed reactions exceed those of simple test-tube analogs by factors of 10^{10} - 10^{20}. In order to account for such a phenomenal effect, model compounds which duplicate one or more of an enzyme's special powers are studied to learn more about the vastly complex protein catalyst. In the belief that the principal enzymatic device is stereopopulation control (near perfect orientation of functional groups), model compounds have been designed and synthesized in which rotation of covalent bonds has been severely restricted. By bringing two functional groups into very close proximity and orientation, rates of reaction comparable to those of enzymes can be achieved in the test tube.

Methods Employed: Spectroscopic methods are used to elucidate the structures of synthetic products and to follow rates of reaction. Various chromatographic procedures are employed for analysis and purification. ^{1}H, ^{13}C and ^{19}F NMR spectra are recorded over the temperature range -100 to +180°C, to detect conformational changes in solution. X-ray crystallography is used to determine conformation in the solid state. Free energies of the various conformers are calculated by use of computer programs (CAMSEQ).

Major Findings: The kinetics of thiolactonization of 3-o-mercaptophenyl-3,3-dimethylpropionic acid gives nonlinear rate plots, the degree of curvature increasing with the time of aging of the alkaline stock solutions. This puzzling phenomenon has been clarified by the observation that the nmr spectrum of the alkaline solution also undergoes a time-dependent change. Electrostatic repulsion between the negative charges in the dianion (S^-, COO^-) drives the normally cisoid conformation of the propionic acid side chain into an extended transoid conformation. After acidification and neutralization of the negative charges, the side chain (7A) slowly reverts to the thermodynamically preferred (and kinetically productive) cisoid conformation (7B)



7A 7B 7C 8

with $t_{1/2}$ = 22 min. Thus, the complex kinetics for thiolactonization must result from the combination of two separate processes: the rapid cyclization of that fraction of substrate which already exists in form 7B and the slow conformational isomerization of 7A to 7B (eq 1). Computer analysis of 70

$$\text{rate} = A \xrightarrow{k_1} B \xrightarrow{k_2} C \quad + \quad B \xrightarrow{k_2} C \qquad (1)$$

kinetic runs has permitted the evaluation of the rate constants, k_1 and k_2, as well as the determination of the cis/trans ratio in each stock solution. The rate constant for the cis ⟶ trans conversion (by nmr) is identical to that for the trans ⟶ cis conversion (by kinetic analysis of ring closure). The barrier to facile rotation (ca. 13 kcal per mole) must arise from the bulk of the sulfur atom, since the analogous phenolic system fails to show a slow conformational change. This is the first example ever reported of an intramolecular reaction with a conformational change as the rate-limiting step. A search for other cases is in progress. The phenomenon has considerable implications in protein chemistry and enzymology, since it serves to demonstrate by simulation that conformational change in a protein, or induced fit by a substrate, can sometimes be slow enough to serve as the rate-limiting step in enzymatic reactions.

We have attributed the extremely high rates of lactonization found for certain phenolic acids (series 8) to rigid cisoid geometries; thus, they may be considered to simulate the enzyme-substrate complex. Other investigators have argued that these very fast ring closures are due, primarily, to relief of overcrowding by ring formation. As part of an effort to generate support for our interpretation, we have now shown that the rates of ring closure are practically the same for X = Cl, Br, or CH_3 in series 8. Since these substituents vary significantly in size, it seems rather unlikely that all the compounds would show the same compulsion to relieve steric crowding. Furthermore, chlorination of the phenolic system (R = X = H) produces isomeric monochloro derivatives in equal amount; hence, the steric environment of either side of the phenol cannot be radically different. Bromination first produces the uncrowded monobromo derivative at low temperature; further bromination gives the dibromo derivative, with the rate constant for the second bromination being even faster than that of the first. Hence, the bromine atom is not rejected because of overcrowding. Finally, nitration of 8 (R = CH_2Ph, X = H) produces isomeric mononitro derivatives and thus, even the nitro group does not encounter a crowded environment.

Significance to Bio-medical Research and the Program of the Institute: Despite the vast amount of research invested in the elucidation of the pathways of specific enzyme-catalyzed reactions, justifications of the rates of these reactions, in comparison with their nonenzymatic counterparts, are still in a primitive state. A sound understanding of the means by which enzymes operate so effectively would lead not only to a better appreciation of biochemical processes in general, but also to a more rational approach to drug design.

Proposed Course: Utilizing the principles of stereopopulation control already developed, additional models will be studied to produce even faster reaction rates than those already observed. The principle will also be extended to other types of reactions, such as hydride transfer and addition/elimination. The control principle will also be used to design conformationally restricted analogs of various drugs with a view to enhancement of activity and reduction of side-effects.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19605-03 LC

PERIOD COVERED

October 1, 1979, through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Chemistry of Substituted Imidazoles

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Louis A. Cohen	Chief, Section on Biochemical Mechanisms	LC NIAMDD
OTHER:	Kenneth L. Kirk	Research Chemist	LC NIAMDD
	Hiroshi Kimoto	Visiting Fellow (Term. April 1979)	LC NIAMDD
	Robert Jerussi	Guest Worker	LC NIAMDD
	Dwight Davis	Staff Fellow (Term. March 1979)	LC NIAMDD
	Olaronge Olubajo	Visiting Fellow (Arr. March 1979)	LC NIAMDD
	Shuji Kondo	Visiting Fellow (Arr. January 1979)	LC NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	2.3	.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Discovery of the widespread and impressive properties of fluoroimidazoles as biological substrates and analogs has provided the impetus for the synthesis of other substituted imidazoles. Novel methods have been developed for the synthesis of previously unknown or difficultly accessible 2-substituted imidazoles. Thus, all 2-halohistamines and 2-halohistidines are now available as well as analogs containing the amino, formyl, cyano, and nitro and azido groups. Discovery of a method for the direct introduction of the 2-amino group (via addition of the imidazole carbanion to phenyl azide) provides a basis for the synthesis of the long sought after 2,4-difluoroimidazoles. 2-Trifluoromethylimidazoles, obtained by another novel route, are readily converted to 2-carboxy, 2-carbethoxy, and 2-cyanoimidazoles. The decarboxylation of 1-methyl-4, 5-dicarboxyimidazole occurs exclusively at C-4, showing the mechanism to involve an ylid rather than carbanion. pH dependence studies show that the decarboxylation of nitroimidazolecarboxylic acids also involves an imidazolium ion, despite the extremely low basicity of the ring.

PHS-6040
(Rev. 10-76)

Project Description: Project No. Z01 AM 19605-03 LC

Development of new methods for the synthesis of fluoro and other analogs of biochemically significant compounds and examination of their properties as enzyme inhibitors and as potential drugs.

Objectives: Fluoro analogs of steroids, purines, pyrimidines and amino acids have been synthesized and tested, several having been found valuable in arthritis and cancer chemotherapy. A larger number have not been prepared because of the limited routes available for the introduction of fluorine. Despite the ubiquitous role of imidazoles in biological systems (RNA, DNA, histidine, histamine, etc.), no fluorinated imidazole had yet been prepared. The initial purpose of this work was to develop a general method suitable for the preparation of fluoroimidazoles, to prepare a series of such compounds, and to test their abilities to serve as enzyme substrates or inhibitors and as replacement for histidine in proteins or for purines in DNA and RNA. Discovery of the widespread and significant biological properties of fluoroimidazoles provided the impetus for the synthesis of other substituted imidazoles.

Methods Employed: Ultraviolet irradiation is used as the key step in the introduction of fluorine. Infrared, ultraviolet, mass, and nmr spectroscopy are used to follow the course of reactions and to elucidate product structure. Various chromatographic procedures are used for analysis and purification of materials.

Major Findings: 2-Trifluoromethylimidazoles (1) are easily obtained by the action of trifluoroacetic anhydride on open-chain precursors, e.g., N,N'-dibenzoylaminoethylenes. Above pH 8, the ring system slowly loses fluoride ion to form a transient difluorodiazafulvene species (2). This highly reactive intermediate reacts with many nucleophiles to generate new functional groups at C-2 of the imidazole ring. Thus, in aqueous ammonia, the trifluoromethyl group is converted to a cyano group (3) and, in water alone, to a carboxyl group. Loss of fluoride ion from 1 requires strong solvation by five water molecules, resulting in the unique phenomenon that the reactions of 1 are linearly dependent on the water content of the medium, rather than on that of the nucleophile. 4-Trifluoromethylimidazoles show a similar property, losing fluoride ion even faster than the 2-isomers. These

properties suggest that trifluoromethyl derivatives of histamine and histidine may serve as irreversible affinity labels for enzyme and receptor sites. To this end, synthetic analogs of LHRF are being prepared containing trifluoromethylhistidines, in the hope that such analogs may combine irreversibly with the LHRF receptor sites. The reactivity of 1 is controlled, in a complex manner, by other imidazole ring substituents and the electronic basis for such control is currently under investigation. [L. A. Cohen, H. Kimoto].

Diazotization of protected 2-aminohistidine and 2-aminohistamine, and treatment of the resulting diazonium salt with azide ion, produces the 2-azido derivatives. Such compounds are of interest, not only as potential competitive inhibitors, but as photoaffinity labels for receptor sites, since the azido group is cleaved to the very reactive nitrene at moderate wave lengths.

N-Triphenylmethylimidazoles react with butyllithium in tetrahydrofuran to generate a carbanion at C-2. Reaction of the carbanion with electrophilic reagents has provided the first syntheses of a variety of new 2-X-histidines and histamines, with X = Cl, Br, I, CHO, COOR, COOH, CH_2OH, and NO_2. These analogs are being screened as potential agonists or competitive inhibitors of the natural imidazoles. Introduction of the nitro group proved to be particularly challenging, since the effective nitrating agents are alkyl nitrates and not nitrites, the normal reagents for such reactions. Equally surprising is the fact that nitration is achieved with the bulky N-triphenylmethyl protecting group, but fails with the much smaller N-methyl group. This method provides the first general synthesis for 2-nitroimidazoles, compounds which have proved to be valuable as antimicrobial and cytotoxic agents, as well as sensitizers for radiation chemotherapy. Synthesis of the histamine and histidine analogs requires elaboration of the side chain of the corresponding 2-X-4-hydroxymethylimidazoles. Current efforts are aimed at the direct introduction of the 2-X substituent into histidine and histamine themselves.

In further studies on the ALP effect in nitrogen heterocycles, kinetic analysis has shown that the decarboxylation of 1-methyl-4-nitroimidazole-5-carboxylic acid follows four distinct pathways, each utilizing a different ionic species. At high pH, decarboxylation of the carboxylate ion proceeds slowly, but no decarboxylation is observed for the isomeric 1-methyl-5-nitroimidazole-4-carboxylic acid. This difference is explained by the fact that the carbanion, which must be generated at C-4, is rendered energetically improbably by electrostatic repulsion of the lone pair at N-3. For analogous reasons, the decarboxylation of 1-methylimidazole-4,5-dicarboxylic acid occurs exclusively at C-5.

Significance to Bio-medical Research and the Program of the Institute: Fluoroimidazoles have already been found to be powerful tools for the investigation of enzyme structure and function, as inhibitors of metabolic pathways and as potential therapeutic agents. We believe that parallel properties will be observed with other analogs of natural imidazoles, possibly with even greater potency. These analogs are designed to answer specific questions

regarding the steric and geometric limitations of natural substrates, the importance of pK_1 and pK_2 of imidazoles to their biological behavior, and the role of ring current effects.

Proposed Course: Continued efforts will be made to develop synthetic routes to difficultly accessible imidazoles. Studies will be continued in an effort to rationalize the anomalous physical and chemical properties of the imidazole series. Many of the compounds will be evaluated for their biological properties.

Publications:

Kimoto, H., Kirk, K. L., and Cohen, L. A.: A Facile Synthesis of 2-(Trifluoromethyl)histamine and 2-(Trifluoromethyl)-L-histidine. J. Org. Chem., 43: 3403-3405, 1978.

Takeuchi, Y., Yeh, H. J. C., Kirk, K. L., and Cohen, L. A.: Adjacent Lone Pair (ALP) Effects in Heteroaromatic Systems. 1. Isotope Exchange of Ring Hydrogens in Alkylimidazoles. J. Org. Chem., 43: 3565-3570, 1978.

Takeuchi, Y., Kirk, K. L., and Cohen, L. A.: Adjacent Lone Pair Effects in Heteroaromatic Systems. 2. Isotope Exchange of Ring Hydrogen in Nitro- and Fluoroimidazoles. J. Org. Chem. 43: 3570-3578, 1978.

Kimoto, H., and Cohen, L. A.: Fluorine Reactivity in 2-Trifluoromethyl-imidazoles. J. Org. Chem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19606-03 LC

PERIOD COVERED
October 1, 1978, through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Halogenated Biogenic Amines in Biochemistry and Pharmacology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth L. Kirk	Research Chemist	LC NIAMDD
OTHER:	Daniele Cantacuzene	Guest Worker	LC NIAMDD
	Cyrus R. Creveling	Research Chemist	LBC NIAMDD

COOPERATING UNITS (if any) L. I. Goldberg, J. Kohli, U. of Chicago; C. Dobsen, F. M. Poulsen, Harvard Univ.; G. Firnau, R. Mishra, McMaster Univ.; P. Seeman, J. Todesco, Univ. of Toronto; J. Martin, Washington Univ.; S. May, Georgia Tech.; D. Murphy, J. Costa, NIMH; D. C. Klein, NICHD.

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Biochemical Mechanisms

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.6	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fluorinated analogs of norepinephrin have been synthesized using the photochemical procedure developed earlier. Studies with these analogs, and fluorinated analogs of tyramine, dopamine, melatonin, and serotonin previously prepared, have revealed important information concerning the mobility of serotonin stored in human platelet vesicles and the use of F^{19} NMR spectroscopy as a probe for molecular motion, the conformational requirements for norepinephrin in interaction with α- and β-adrenergic receptor systems, and the role of phenol pK and lipophilicity in determining activity of biogenic amines.

-S-6040
(Rev. 10-76)

Project Description:

Development of new methods for the synthesis of halogenated analogs of biogenic amines, study of their physical properties, and evaluation of their behavior in biological systems as antagonists or agonists.

Objectives: Halogenated analogs have proved to be powerful tools for the study of transport, storage, release, and action of biogenic amines, since they simulate the geometry of the natural compounds so well. Continued studies should provide significant information about the roles of these essential metabolic species and possibly lead to therapeutic use.

Methods Employed: Ultraviolet irradiation and various chemical methods are used for the introduction of halogen into biogenic amines. Infrared, ultraviolet, mass, and nmr spectroscopy are used to follow the course of reactions and to elucidate product structure and purity. Various chromatographic procedures are used for purification and analysis of materials. Electron energy-loss spectroscopy is used to detect and localize fluorine-containing materials in tissues.

Major Findings: The three ring-fluorinated analogs of dopamine (FDA) have been synthesized and subjected to biological evaluation: 2-FDA, 5-FDA and dopamine (DA) are equipotent as DA vascular receptor agonists, on the basis of the increase in renal blood flow of phenoxybenzamine-treated dogs, while 6-FDA is ca. 4-fold less potent. The three analogs are comparable to DA in α-adrenergic agonist potency, as measured (in vivo) by femoral blood flow in the dog; similar results are obtained for β1-adrenergic agonist activity, as measured by in vivo cardiac contractural response. Response in the central nervous system (CNS) was determined by comparing the abilities of 2-, 5-, and 6-FDA, relative to DA, to displace specific radioligands in brain slices. Again, 2-FDA, 5-FDA, and DA were equipotent, with 6-FDA showing some diminished activity. Thus, it would appear that the presence of fluorine at the 2- or 5-position of the catechol ring has no significant effect on biological activity, while the 6-fluoro analog is somewhat weaker in two of the three tests.

The dramatic differences in agonist specificities previously observed for the isomeric ring-fluorinated norepinephrins (FNE) have been investigated in greater detail. Peripheral studies (in vitro) have confirmed that 5-FNE, 6-FNE, and NE are equipotent as α-adrenergic agonists (guinea pig aorta), while 2-FNE has only marginal activity. 2-FNE and NE are equipotent as β-adrenergic agonists, 5-FNE is ca. seven times more potent, while 6-FNE is essentially inactive. In vivo experiments agreed with the in vitro results. Thus, the effects of 2-, 5-, and 6-FNE on cardiac contractural force (β1-adrenergic response), arterial blood pressure (β2-adrenergic response), and femoral blood flow (α-adrenergic response) were compared to those of NE in anesthetized dogs. The results demonstrated that 2-FNE acts on β1 and β2 adrenergic receptors, 5-FNE on α and β1 receptors, and 6-FNE only on α-receptors.

Extension of these studies to the CNS was accomplished by examining the fluoro analogs in the cyclic-AMP generating system in rat brain slices. The results show that the 2-FNE response corresponds to that of the β-component (propranolol-sensitive) of the system, while 6-FNE gives a response corresponding to that of the α-component (phentolamine-sensitive). 5-FNE behaves similarly to NE (α- and β-responses). The effects of FNE's on binding of radioactive α- and β-adrenergic antagonists to rat cerebral cortical membranes confirm the selectivities of their action in the CNS, and suggest that the specificities may result from binding selectivity. [K. L. Kirk, D. Cantacuzene, C. R. Creveling, J. Daly, D. McColloh, Y. Nimitkitpaisan, W. Padgett, E. McNeal, L. I. Goldberg (U. Chicago) and J. D. Kohli (U. Chicago)].

The value of the ring-fluorinated biogenic amines as pharmacological tools has been amply demonstrated. In addition, correct interpretation of the causes of the specificities observed may give insight into the nature of specific receptor-agonist interactions and/or receptor structures. Physical chemical studies on the isomeric FNE's, designed to determine the molecular effects of fluorine substitution, are in progress. Analogs with additional structural modifications are being prepared to examine the relationships of fluorine substitution to other structural parameters which have already been identified. Thus, α-methyl-FNE's and N-isopropyl-FNE's (fluoroisoproteronol) have been synthesized for adrenergic agonist specificity studies. The corresponding ring-fluorinated DOPA's are being prepared for in vivo CNS experiments.

Melatonin (N-acetyl-5-methoxytryptamine) is a potent inhibitor of luteinizing hormone-releasing factor (LHRF). Hydroxylation of melatonin at the 6-position destroys its activity toward LHRF. 6-Fluoro- and 4,6-difluoromelatonin have been synthesized and investigated in pituitary glands with the hope that the stronger C-F bond would delay inactivation, resulting in a longer lasting drug. Initial results indicate that 6-fluoromelatonin and melatonin are equipotent in inhibition of LHRF, while 4,6-difluoromelatonin is 0.03 times as active. In vivo studies show that all three compounds have virtually identical biological half-lives. [K. L. Kirk, J. E. Martin (Washington U.) and D. C. Klein (NICHD)].

F^{19} NMR Spectra as Probes of Molecular Motion

F^{19} NMR studies of fluorinated molecules in intact cells have continued. Within blood platelets are found membrane-enclosed granules which contain high concentrations of 5-hydroxytryptamine (5-HT), along with adenine nucleotides and divalent metal ions. F^{19} NMR spectra of fluorinated-5-HT, incorporated into the granules of both human and pig platelets, have shown that the motional state of the 5-HT is restricted. On the basis of P^{31} NMR data (adenine nucleotides), it would appear that high molecular weight aggregates are formed between the nucleotides and metal ions, and that these aggregates further bind 5-HT,

restricting its motion. [K. L. Kirk, J. L. Costa (NIMH), C. M. Dobson (Harvard U.), F. M. Poulsen (Harvard U.), C. R. Valeri and J. J. Vechione (Naval Blood Research Lab., Boston)].

Significance to Bio-medical Research and the Program of the Institute
Biogenic amines play a key role in neurotransmission, metabolism, and in control of various physiological processes. Relatively little is known regarding mechanisms of transport, storage, release, and action of these agents. The availability of effective analogs provides a unique set of tools for the study of these mechanisms.

Proposed Course: Additional analogs will be synthesized to help answer specific questions regarding the biochemistry and pharmacology of biogenic amines. Evaluation of the analogs will be continued with a view to development of possible therapeutic agents.

Publications:

C. R. Creveling, H. Seifried, D. Cantacuzene, and K. L. Kirk: Fluorine-Substituted Catecholamines as Substrates of Catechol-O-Methyltransferase. Transmethylation, eds. Usdin, Borchardt, Creveling; Elsevier, New York, 1979, pp. 269-276.

J. L. Costa, C. M. Dobson, K. L. Kirk, F. M. Poulsen, C. R. Valeri, and J. J. Vecione: Studies of Human Platelets by ^{19}F and ^{31}P NMR. FEBS Letters 99, 141-145 (1979).

J. L. Costa, C. M. Dobson, K. L. Kirk, F. M. Poulsen, C. R. Valeri, and J. J. Vechione: Nuclear Magnetic Resonance Studies of Blood Platelets. Proc. Royal Soc., Series B (in press).

J. E. Martin, K. L. Kirk, and D. C. Klein: Effects of 6-Hydroxy-, 6-Fluoro-, and 4,6-Difluoromelatonin on the In Vitro Pituitary Response to Luteinizing Hormone-Releasing Hormone. Endocrinology (in press).

D. Cantacuzene, K. L. Kirk, D. H. McCulloh, and C. R. Creveling. Effect of Fluorine Substitution on the Agonist Specificity of Norepinephrin. Science, 204, 1217-1219 (1979).

K. L. Kirk, D. Cantacuzene, Y. Nimitkitpaisan, and C. R. Creveling. Synthesis and Biological Properties of 2-, 5-, and 6-Fluoronorepinephrins. J. Med. Chem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19801-34 LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Service Functions and Instrumentation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. F. Johnson	Section Chief	LC-NIAMDD
Other:	H. J. C. Yeh	Research Chemist	LC-NIAMDD
	W. R. Landis	Chemist	LC-NIAMDD
	P. Parisius	Chemist	LC-NIAMDD
	B. Baer	Chemist	LC-NIAMDD
	A. Wong	Chemist	LC-NIAMDD
	N. Whittaker	Chemist	LC-NIAMDD
	B. Torain	Biol. Lab. Tech.	LC-NIAMDD

COOPERATING UNITS (if any)

NONE

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Laboratory of Chemistry

SECTION
Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.0	7.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are determined on a quantitative basis, using ultramicro, micro, and semi-micro techniques as required. The materials analyzed include organic and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents when requested. Instrumental analyses include: GC/MS spectrometry, gas-liquid chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, amino acid analysis, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request.

PHS-6040
(Rev. 10-76)

Project Description: Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are determined on a quantitative basis, using ultramicro, micro, and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents when requested. Instrumental analyses include: GC/MS spectromometry, gas-liquid chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, atomic absorption spectrometry, amino acid analysis, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request.

During the past year the total number of analyses performed in the microanalytical laboratory was 12,866, an increase of 21% since the last reporting period due primarily to our new service of protein analysis. Review of the special types of analyses are shown below:

	Analyses Run		
	1977-78	1978-79	% Change
Carbon	2345	2398	2.3
Hydrogen	2345	2398	2.3
Nitrogen	4541	3886	-14.4
Phosphorous	337	310	- 8.0
Sulfur	87	88	0.0
Halogens	346	270	-22.0
Metals	201	36	-82.1
Weight Loss	65	45	-30.8
Rotations	55	35	-36.4
Miscellaneous	390	328	-15.9
Protein (Technicon)		3072	
	10,712	12,866	+21.0

Under a continuing contract with Galbraith Laboratories 5 special samples were submitted for a total of 6 analyses. The distribution of service rendered was LC/LBC (58%); NIH, not including LC/LBC (37%); and outside agencies (5%). This distribution is comparable to the previous reporting period.

During the past year an amino acid analysis service has been instituted in the Section. Over the past 6 months Ms. Barbara Torain has run a total of 279 samples (206 protein hydrolysates -73 physiological). The requests by Institute was NIAMDD (55), NCI (2), and NHLI (2). Our first Beckman 121 MB was acquired by transfer from the Division on Intramural Research - Hypertension Endocrine Branch. A second instrument has just been obtained from the laboratory of Dr. Geroge Glenner, Laboratory Experimental Pathology, NIAMDD and is in the process of complete overhaul at a small fraction of the expense of a new

instrument. The two analyzers give us the capability of 0.1 to 10 nanamoles and 10 to 25 nanamoles respectively.

It appears that this new service will obviate the problem of occasional analyses for a large number of investigators and the purchase of expensive equipment for such occasional use.

The NMR service includes a Varian A-60, HA-100, and JEOL FX-100 pulse Fourier transform spectrometers. As in the last reporting period approximately 150 spectra were run on the A60 by Mr. Benjamin Miller and over 1000 by individual investigators. Mr. Miller continues to maintain the documentation, ordering, and stocking of organic chemical stores for Building 4. A total of 520 spectra were run on the JEOL FX-100 as a service function by Dr. Herman Yeh in addition to 75 on a Varian HR-200 (located in a different Section). These specialized spectra were in addition to Dr. Yeh's research program, reported elsewhere. This represents a 36% increase of service workload for Dr. Yeh.

More than 1000 samples were submitted to Mr. William Landis for mass spectral analysis on the Hitachi Perkin-Elmer RMU-6E. Analyses involved several spectral determinations. Each sample analysis was successfully completed according to the investigator's requests and the instrument's capability. This involved standard qualitative spectra; isotope quantitative mass-ratio measurements; on-scale spectra recorded either/or the visicorder and the pen recorder; and sample mixtures requiring fractional volatility-changing rate separations occurring in the solids-inlet probe.

The instrument was interfaced with the newly-acquired VG Data System. We will now be able to perform rapid repetitive scanning, plus high resolution determinations in accordance with the design limitations of the Hitachi mass spectrometer.

The number of samples analyzed are tabulated as follows:

NIAMDD - Laboratory of Chemistry	1037
NIH, other than NIAMDD-LC	101
Outside NIH	66
(Total samples analyzed)	1204

Mr. Noel Whittaker is the principal operator of the Finnigan GC/MS system. A heavy sample load of over 1000 solid probe samples were run, utilizing both chemical ionization and electron impact modes. This system is used primarily for investigators of the LC (85%), but there is a small volume of samples from other institutes (10%) and outside the NIH (5%). Mr. Whittaker is in the process of checking the performance specifications of our recently acquired VG 7070 exact mass, double focusing GC/MS spectrometer. This instrument will significantly upgrade the mass spectrometer capabilities of the Section.

Evaluation is in progress of a new microwave plasma detector system that is reported to allow for simultaneous elemental analysis of GC peaks for empirical formula determination. Feasability of purchase is being studied.

The Section Chief continues to serve as Project Officer on an NIAMDD contract with the Medical Research Council of England. This contract provides reference steroid compounds to qualified research workers in the U.S.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19802-06 LC

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Characterization of Dehydrogenase-Isomerase Enzymes and their Metabolic Pathways in Tetrahymena Pyriformis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Nancy Lamontagne	Research Chemist	LC-NIAMDD
Other:	David F. Johnson	Section Chief	LC-NIAMDD

COOPERATING UNITS (if any)

Chester E. Holmlund , Dept. of Chem., Univ. of Md., College Park, Md.

LAB/BRANCH Laboratory of Chemistry

SECTION Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

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1.5	1.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our objective is to characterize the nature of a dehydrogenase-isomerase system of T. pyriformis by purification of the dehydrogenase. We will study the substrate specificity and kinetics of the partially purified enzyme. A growth inhibition study will also be done to investigate the hypothesis that these transformations are related to the toxicity of steroids to this protozoan.

Project Description: The protozoan Tetrahymena pyriformis has steroid dehydrogenases that are similar in functional properties to the dehydrogenases of higher eucaryotes. Specifically, our prior identification of 4-androstene-3β,17β-diol and pregnenolone as transformation products from the incubation of T. pyriformis with testosterone and progesterone, respectively, indicated that this protozoan possesses a dehydrogenase-isomerase system that can isomerize a Δ^4-steroid to a Δ^5-steroid and reduce the C-3 carbonyl to a 3β-hydroxyl. The characterization of this system is being pursued in several ways.

The β-hydroxysteroid dehydrogenase (β-HSDH) is obtained as a crude supernatant from a 100,000 x g centrifugation of ruptured cells. Purification of the supernatant involves chromatography on a DEAE cellulose column, ammonium sulfate precipitation, and affinity chromatography on blue sepharose. Although the amount of this enzyme activity in T. pyriformis is small, the development of a quick spectrophotometric assay and availability of large fermentors is making the purification feasible. Once a sufficient amount of β-HSDH activity is partially purified it will be used for kinetic and substrate specificity studies.

Preliminary data indicate that the partially purified β-HSDH is specific for the 3β-hydroxyl of a steroid, but the presence of that functional group is not sufficient for binding. Although chlosterol was 100 to 1,000 times more reactive than 4-androstene-3β,17β-diol with the partially purified β-HSDH, there was no response to cholesterol with a more purified preparation. Thus it is possible that T. pyriformis possesses more than one 3β-HSDH. Further substrate specificity studies will be done to determine the structural requirements for activity of a 3β-hydroxysteroid with a purified preparation of β-HSDH.

Cultures of T. pyriformis will be grown in the presence of varied concentrations of unlabeled steroids. Growth inhibition will be measured by cell counting in a hemocytometer. A fixative technique has been developed to immobilize the protozoa and trypan blue will be used to distinguish between viable and non-viable intact cells.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19803-06 LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Applications of NMR in Biochemical and Biological Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. J. C. Yeh	Research Chemist	NIAMDD-LC
Others:	D. F. Johnson	Section Chief	NIAMDD-LC
	S. Chiang	Visiting Fellow	NIAMDD-LC
	F. Hayashi	Visiting Fellow	NIAMDD-LC
	Y. Takeuchi	Visiting Fellow	NIAMDD-LC
	K. L. Kirk	Research Chemist	NIAMDD-LC
	L. A. Cohen	Section Chief	NIAMDD-LC

COOPERATING UNITS (if any) D. D. Dominquez and M. F. King (George Wash. Univ.), T. Axenrod and M. J. Wieder (City Col. of City Univ. New York); T. Khin and G. A. Webb (Univ. of Surrey, England); S. Bulusu (U.S. Army Armament Res. and Dev. Command); C. C. Yang and S. M. Wang (Nat. Tsing Hua Univ., Taiwan).

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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop nuclear magnetic resonance methods for elucidating molecular structure and for studying the interactions within and between molecules, especially those of biological interests.

Project Description: Basic information on the structure and molecular interactions of biologically important materials is of significance to biochemical and biological research. One of the most promising approaches in obtaining such information is through the use of nuclear magnetic resonance. The purpose of this project is to develop and apply nuclear magnetic resonance methods for studying molecular structures and molecular interactions in biochemical and biological systems.

Many of the biologically significant functions of metalloporphyrin-containing proteins are directly related to the interactions between the central metal and the porphyrin nitrogens and between the central metal and coordinating ligands. Such interactions should be very suitable for investigation by various NMR techniques. Although several recent communications have successfully demonstrated the use of N-15 NMR spectroscopy as a means of assessing structural information about porphyrin, metalloporphyrins, and related ring systems, relatively few investigations of these systems by means of metal nuclide NMR have been reported. However, a systematic study of N-15 and/or metal nuclide NMR spectroscopy of the metal-ligand and metal-porphyrin interactions in these molecules remains to be explored. We have made such systematic study of the effects of ligands on these interactions in doubly labeled cadmium meso-tetraphenylporphyrin [^{111}Cd-TPP($^{15}N_4$)] in order to examine the utility of these nuclei in obtaining useful information about these systems. To examine the effect of ligands on the Cd-111 or N-15 chemical shift as well as the Cd-N coupling constants, the ^{111}Cd-TPP($^{15}N_4$) was treated with a series of substituted pyridines. Our results indicate that the N-15 chemical shift is insensitive to the ligand effect and therefore it is not practical to use N-15 NMR as a tool for probing the interaction of ligand with metalloporphyrin. A relatively large Cd-111 chemical shift difference was observed for the series of ligands (∿17 ppm). The result suggests that Cd-111 NMR (and probably the other metal nuclides) is a better tool for probing the interaction of ligands with metalloporphyrins. A good correlation between the Cd-111 chemical shift and the pK_a values of the substituted pyridine ligands is observed. An extension of this work to other metal nuclides is in progress.

As a part of NMR studies on metalloporphyrin-containing molecules, we have undertaken a C-13 NMR study on the conformation and structure of hemoglobin which will be ^{13}C-labeled specific at the histidine residues. Work on the preparation of the ^{13}C-labeled hemoglobin is now in progress.

As a part of the project in the use of NMR for structural elucidation, various NMR methods have been employed successfully to assign the signals for H-4 and H-5 in alkylimidazoles and to probe the ring substituent effects of a series of anilines and anilinium ions.

Hemerythrin, a non-heme oxygen-carrying protein found in the erythrocytes of certain marine invertebrates, normally occur in an octameric form of molecular weight 108,000, and can be dissociated into the monomeric species in the presence of several organic mercurials. Each subunit contains two atoms of iron and can reversibly bind one molecule of oxygen. Oxidation of either oxyhemerythrin or deoxyhemerythrin gives the iron(III) species, methemerythrin, which coordinates a number of simple anionic ligands to give a series of characteristic methemerythrin complexes.

We have made comparative NMR studies of the pH-dependent chemical shifts of histidine C-2 protons in the spectra of P. gouldii hemerythrin A and hemerythrin B, and T. pyroides myohemerythrin in their metazide forms in order to obtain local structural information of these proteins. By comparing their NMR spectra in the histidine C-2 proton resonance regions, we are able to assign two histidine resonances in hemerythrin A (His-82 and His-34) and one each in hemerythrin B (His-34) and myohemerythrin (His 57). The readily titrable His-82 (pK 6.5) in hemerythrin A and His-57 (pK 6.4) in myohemerythrin in the pH range 4 to 9.5 suggest that these histidines probably exposed near the surface of the protein. That His-34 in hemerythrin A and hemerythrin B can be observed only below pH 4.5 indicates that His-34 may be partially buried inside the protein or hydrogen-bonded to nearby amino acid residues. The results eliminate the possibility of His-32, His-34 and His-57 as ligands to the iron atoms and hence imply that a maximum number of five histidines are coordinated to the iron atoms.

We also examined the histidine C-2 proton titration behaviors of both octameric and monomeric methemerythrins with several anions (e.g. N_3^-, Cl^- and CN^-). The titration data for His-34 were found noticeably different between ligands such as N_3^- and Cl^- (or CN^-), and the pK_a values of His-34 were found considerably higher for the monomeric than the octameric methemerythrins. These findings tend to suggest that the local environment of His-34 is different for these methemerythrin ligand complexes and also suggest the involvement of His-34 in the intersubunit interactions. An extension of this work to other ligands is planned.

Neurotoxins from snake venoms are a group of small proteins which block the neuromuscular transmission at the post-synaptic membrane by high affinity binding to acetylcholine receptor and accordingly have been extensively studied in the field of neurochemistry. The toxins show a high degree of sequence homology and 13 amino acid residues are found to be invariant among more than 50 neurotoxins being investigated. The roles played by some of the amino acid residues in the binding to the receptor and in the lethal activity of the toxins have been the subject of considerable interest.

Basic information on the conformation and structure of the toxins in solution is of importance to the understanding of the nature of toxin-receptor interaction. Although the main chain conformation of these proteins has been elucidated in some detail, relatively few investigations on the local structure of the amino acid residues of the toxins have been reported. In order to gain understanding of the structure-toxicity relationship for the toxins, a possi-

bility of using NMR to obtain the detailed strucutral information of these proteins has been recently explored. The preliminary results of NMR work on cobrotoxin and pelamitoxin have revealed some detailed structural information, including the microenvironments of His-4, Tyr-25 and His-32 of cobrotoxin and His-31 and His-54 of pelamitoxin. A detailed structural study of these toxins, including some chemical modifications, is now in progress.

Publications:

Dominguez, D. D., King, M. M. and Yeh, H. J. C.: A NMR study of metal-ligand interaction in doubly labeled ^{111}Cd meso-tetraphenylporphyrin($^{15}N_4$). J. Mag. Resonance 32: 161-165, 1978.

Takeuchi, Y., Yeh, H. J. C., Kirk, K. L. and Cohen, L. A.: Adjacent lone pair (ALP) effects in heteroaromatic systems. 1. Isotope exchange of ring hydrogens in alkylimidazoles. J. Org. Chem. 43; 3465-3570, 1978.

Axenrod. T., Wieder, M. J. Khin, T., Webb, G. A., Yeh, H. J. C. and Bulusu, S.: ^{13}C NMR: Substituent effects on ore-tcnd ^{15}N-^{13}C coupling constants in anilines and anilinium ions. Org. Mag. Resonance 12: 1-4, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19804-06 LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Development of Chemical Methods and Compounds for the Study of Biological and Medical Problems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Calvin M. Foltz	Research Chemist	NIAMDD-LC
Other:	Lance A. Liotta	Expert/Pathologist	NCI-LP

COOPERATING UNITS (if any)

National Cancer Institute

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Microanalytical Services and Instrumentation

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NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The primary goal of this work is to contribute to the investigation and solution of basic biological and medical problems. This is to be done by the application of chemistry to the study of the structures of biomolecules, by the synthesis and study of chemical compounds, and by the study of the interactions of chemicals with cells, such as T241 sarcoma cells and B16 melanoma cells, in vitros and with tumor cells and tumors in C57B1/6N mice and nude mice.

Areas of special interest are organic chemistry, photochemistry, selenium chemistry, biochemistry, medicinal chemistry, tissue culture, and cancer chemotherapy.

The objective of this project is to make contributions to the investigation and solution of basic biological and medical problems by the application of chemical, physical, and biological methods.

Work is in progress on the study of cancer metastases in the mouse. The purposes of this study are (a) to increase our understanding of the biochemical mechanisms operating in the different stages of the formation of metastases, (b) to study the effects of chemicals on metastases and their development, and (c) to apply the knowledge obtained thereby to the design and synthesis or organic compounds of potential use in inhibiting the formation of metastases or selectively destroying them in human beings.

The findings of these studies may also contribute to the study of other biological and medical problems.

The effects of selected chemicals on metastases and their development in C57BL/6N are being studied following administration of cells of the T241 sarcoma or B16 melanoma intramuscularly, subcutaneously, on intravenosly.

The population of cells in most malignant tumors is thought to be heterogeneous. If the subpopulation of the cells of a given tumor which accounts for the tumor's invasiveness and metastatic potency could be isolated from the primary tumor the study of such a cell line might reveal some new approaches to interference with the process of metastasis formation or to agent lethal to the metastatic cells. The isolation of a subpopulation of cells of high metastatic potential from the T241 sarcoma is being attempted by studying the binding of tumor cells to collagens I & IV and by soft agar techniques. The cell lines isolated from the B16 melanoms by I. Fidler are also being studied.

A neutral protease has been isolated from the highly invasive and metastatic mouse sarcoma, T241, and partially purified. This enzyme preferentially degrades basement membrane collagen and may play a role in the invasiveness and metastatagenicity of the tumor. Experiments are in progress to determine whether the partially purified enzyme can provoke an immune response capable of interfering with tumor invasiveness and formation of metastases. If antibodies to the enzyme can be produced in another species, they will be useful in studying the distribution of the enzyme and its biological role. Using two types of assays employing cells of T241 sarcoma and B16 melanoma cell lines and media obtained from cell cultures, a correlation has been found between the incidence of spontaneous metastases and the kind of degradative activity displayed by this enzyme. If this enzyme does play a critical role in tumor invasiveness and metastasis formation, it may afford a route to interference with the process.

Publications: Schwarz, K. and Foltz, C.: Selenium as integral part of factor 3 against dietary necrotic liver degeneration. J. Amer. Chem. Soc. 79; 3292-3293, 1957.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 19806-04 LC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Nature of Steroid-Receptor Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. S. Simons, Jr.	Senior Staff Fellow	NIAMDD-LC
Other:	D. F. Johnson	Section Chief	NIAMDD-LC
	M. Pons	Visiting Fellow	NIAMDD-LC

COOPERATING UNITS (if any)
E. I. B. Thompson, NCI
Ruth Doherty, Univ. of Md. Chem. Dept.

LAB/BRANCH
Laboratory of Chemistry

SECTION
Microanalytical Services and Instrumentation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

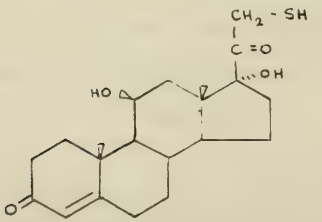
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to define the initial, intra-cellular events of steroid hormone action. Synthetic glucocorticoid derivatives, some of which could react to form covalently labelled receptors via affinity labelling, are being used with glucocorticoid-responsive rat hepatoma tissue culture cells to examine: (1) steroid-receptor binding site interactions; (2) the effects of steroid binding on receptor conformation; and (3) the nature of "activation" of receptor-steroid complexes. A novel group of potential affinity labelling steroids, which may yield a functionally active, covalently labelled receptor, appear promising. Fluorescence spectroscopy represents another technique for examining these interactions. We have developed a new affinity labelling technique called fluorescent chemo-affinity labelling (FCAL) which potentially could give covalent, fluorescent receptor-steroid complexes. Finally we have determined that a class of anti-inflammatory steroids, predicted to be inactive glucocorticoids, are among the most potent glucocorticoids yet described.

Project Description: Steroid specific events are induced by nanomolar concentrations of hormone in responsive cells. Thus, extremely sensitive methods are required in order to monitor the steroid at physiological levels. Fluorescence spectroscopy can 1) detect some compounds at concentrations as low as 10^{-12}M and 2) provide information about the microenvironment of the fluorescent molecule. These two properties make fluorescence spectroscopy ideally suited for a study of steroid-receptor interactions. Due to the tendancy of receptor-steroid complexes to dissociate, a covalently bound fluorescent receptor-steroid complex would be even more useful. In order to obtain such a complex, we have developed a new methodology for affinity labelling. We call this method chemo-, or chemically activated, affinity labelling since the addition of a specific chemical is required to initiate the affinity labelling process. Chemo-affinity labelling (CAL) thus combines the ability to control the timing of covalent bond formation, a distinguishing feature of photo-affinity labelling (PAL) with the advantages of the relatively stable and specific "reactive" functional groups found in conventional, or electrophilic, affinity labelling (EAL). Our chemical initiator for the chemo-affinity labelling process is o-phthalaldehyde (OPTA), which should also be useful as a new bi-functional, chemical cross-linking reagent. OPTA rapidly reacts with primary amines and thiols to give 1-alkylthio-2-alkylisoindoles in high yield (Simons, S. S., Jr. and Johnson, D. F. (1978) J. Org. Chem., 43, 2886-2891). [In collaboration with Ruth Doherty of Univ. of Md., we are trying to obtain an x-ray structure of an isoindole derivative in order to unambiguously prove the isoindole structure and to answer some basic theoretical questions about this ring system.] Since these isoindoles are usually intensely fluorescent (Simons, S. S., Jr. and Johnson, D. F. (1978) Anal. Biochem., 90, 705-725), this chemo-affinity labelling reaction has been named fluorescent chemo-affinity (FCA) labelling, or FCAL. In order to obtain a fluorescent, covalent receptor-steroid complex, we have prepared three hydrolytically stable glucocorticoid derivatives (1-3) which should participate in the FCAL reaction. In model



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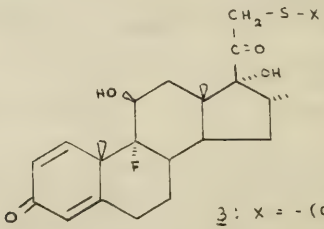


3 : X = $-(CH_2)_4-SH$

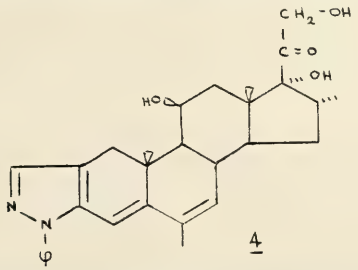
4 : X = $-(CH_2)_3-NH_2$

studies with OPTA and an added amine or thiol, each steroid gives a good yield of the desired isoindole, two of which (derived from 2 and 3) are strongly fluorescent and display good stability and response to changes in solvent polarity. In collaboration with Dr. Thompson, each steroid displayed high affinity for the glucocorticoid receptors from rat liver hepatoma tissue culture (HTC) cells and induced the enzyme tyrosine aminotransferase (TAT). Thus these

synthetic steroids show excellent promise for being effective FCA labels for glucocorticoid receptors.

We have previously reported studies (in collaboration with Dr. Thompson) on two novel cortisol derivatives (cortisol-21-thiol acetate and -21-(O-ethyl) xanthate) which potentially could transfer a small functional group (i.e., acetyl or thionocarbethoxy) to the glucocorticoid receptor to give a functionally active, covalently labelled receptor. Since we have been unable to obtain the necessary radio-labelled cortisol derivatives, we have further examined these steroids by comparing their properties to that of the expected cleavage product, cortisol-21-thiol. The relative affinities of these steroids for receptors did not correlate with the bulk of the steroid substituents since the thiol, thiol acetate and xanthate derivatives were all approximately equal in being better competitors of ^{3}H-dexamethasone binding than the parent steroid cortisol. Interaction of the latter two steroids with HTC cell receptors was further examined in a cell-free exchange assay that renders steroid-free receptors incapable of binding the subsequently added ^{3}H-steroid. The behavior of the chemically reactive thiol acetate and xanthate derivatives in this exchange assay, their unexpectedly high apparent affinity for receptors, and the similiarity of their cell-free and whole cell properties with those of cortisol-21-thiol are all consistent with the reaction of cortisol-thiol acetate (or -xanthate) with the receptor to yield a functionally active, covalently labelled glucocorticoid receptor and cortisol-thiol.

In an effort to discover steroids with even higher affinities for receptor that could be used in the above studies, we examined the pyrazolo-steroid 4, which is an extremely potent anti-inflammatory steroid but was predicted to



4

to be an inactive glucocorticoid on the basis of structure-activity data. We have now shown that 4 is also a potent glucocorticoid in a well defined system. The observed high affinity of 4 for the HTC cell receptors indicates that the current concepts of glucocorticoid structure-activity should be revised. The close correlation of biological activity of 4 in anti-inflammatory and TAT induction assays suggests that the latter assay using the HTC cells, might be a much more convenient and rapid screening test for anti-inflammatory steroids than the presently used, more cumbersome assays. Furthermore, the close correlation between the two assays supports the hypothesis that both glucocorticoid induced responses are mediated by specific steroid receptors.

We are applying organic chemistry to the study of an important biological molecule(s) - the intracellular steroid hormone receptor(s). A molecular understanding of steroid-receptor interactions is highly desirable, especially since the therapeutically valuable, steroid-specific differences are most likely expressed at this level of steroid hormone action.

Publications:

Simons, S. S., Jr. and Johnson, D. F.: Reaction of o-phthalaldehyde and thiols with primary amines: Formation of 1-alkyl (and aryl)thio-2-alkylisoindoles. J. Org. Chem., 43, 2886-2891 (1978).

Simons, S. S., Jr. and Johnson, D. F.: Reaction of o-pthalaldehyde and thiols with primary amines. Fluorescence properties of 1-alkyl(and aryl)thio-2-alkylisoindoles. Anal. Biochem., 90, 705-725 (1978).

Simons, S. S., Jr., Thompson, E. B. and Johnson, D. F., "Anti-inflammatory pyrazolo-steroids: Potent glucocorticoids containing bulky A-ring substituents and no C_3-carbonyl." Biochem. Biophys. Res. Comm., 86, 793-800 (1979).

Simons, S. S., Jr.: Factors influencing association of glucocorticoid receptor-steroid complexes with nuclei, chromatin and DNA: Interpretation of binding data. In Baxter, J. D. and Rousseau, G. G. (Eds.): Mechanisms of Glucocorticoid Hormone Action. Springer-Verlag, in press.

ANNUAL REPORT
LABORATORY OF EXPERIMENTAL PATHOLOGY

Studies in the Section of Molecular Pathology (Dr. George G. Glenner and staff) are concerned with various apsects of the pathogenesis of amyloidosis. Work is proceeding in four main areas:

1. Isolation and chemical characterization of amyloid fibril protein from various types of systemic amyloidosis and localized amyloid deposits. This includes patients with Alzheimer's syndrome (presenile dementia) where a mechanism for the pathogenesis of associated localized amyloid despsits has been proposed.

2. Mechanism of formation of amyloid deposits from serum precursors. Work in this area involves studies in inbred stains of mice which are either highly suseptable or resistant to the deposition of amyloid following stimulation by endotoxin.

3. Isolation and purification of proteolytic enzymes involved in amyloid fibril formation. This is closely related to the two preceeding areas of research and involves studies on clevage of neurofilamentous proteins by lysosomal enzymes and the deposition of the products of this reaction in the tissues.

4. Immunochemical characterization of amyloid fibrils from different syndromes in tissue sections for diagnostic and therapeutic purposes. A large group of amyloid fibril proteins have been shown to possess as their major protein component some portion of an immunoglobulin light polypeptide chain. Others have been associated with additional serum precursors (SAA). It is hoped that further immunochemical characterization of these may lead to the development of antisera which can be used for diagnosis of amyloidosis. Development of a therapy for amyloidosis would also be aided by further understanding of the structure of amyloid.

Additional work on amyloid is being carried out by Dr. Jean D. Sipe. She has been working on acute phase reactants and amyloidosis. Serum amyloid A (SAA) is an acute phase reactant that was detected within the past decade by its cross-reaction with antibodies raised to denatured amyloid A fibrils. The original and structure of SAA and its role in the pathogenesis of secondary amyloidosis has been investigated, employing the mouse as an experimental model. Induction of the acute phase SAA response on inflammatory agent such as endotoxin has thus far been observed only in vivo. Dr. Sipe has shown that two cell populations are involved in the acute phase SAA response to lipopolysaccharide (LPS). A macrophage response to LPS gives rise to SAA inducer, which in turn stimulates SAA synthesis by liver and possibly other tissues.

Three stages in the SAA response to LPS and other agents of inflammation have been observed: a latent period of 2 to 3 hr during which the SAA concentration remains at baseline values and in which SAA inducer appears. The period of SAA synthesis which lasts for 24 hr past induction, and the

period of deinduction during which SAA synthesis ceases and SAA concentration returns to baseline values by 48 hrs after induction.

The following studies have been conducted in the Section of Cytogenetics (Dr. Tjio and staff) during the past year.

1. Murine cytogenetic disorders. The appearance of naturally occurring thymocytotoxic autoantibodies (NTA) and spontaneously produced antibodies to single-stranded DNA (ssDNA) are characteristic of the autoimmune disease of NZB mice. These autoantibodies were found to be inherited traits, but influenced by sex hormones. In F_1 hybrids of the NZB strain and a normal strain, it was found that testosterone suppressed the formation of NTA. However, in the parent NZB strain no sex difference or suppression by testosterone of NTA production was observed. From various crosses they postulate an androgen insensitivity in NZB mice. In support of such a concept, in preliminary work with Dr. Donald Shreffler, Washington Univ., St. Louis, they have studied a serum protein (Slp) which is also testosterone sensitive. Most strains show a sex difference with regard to slp production but NZB animals do not. They are currently studying this in greater detail to determine if androgen insensitivity is related to autoimmune disease.

2. Murine cytogenetic studies. Experiments in collaboration with Dr. A. Steinberg and D . J.H. Tjio (NIAMDD) to determine the effects of genetic background on the expression of hyperdiploidy in various crosses with NZB mice are currently underway. They have completed analysis of crosses of the NZB strain with the normal DBA/2 strain and found that hyperdiploidy was inherited as a recessive trait and not related to the presence of other immunological abnormalities including splenomegally, hypergammaglobulinemia, spontaneous antibodies cytotoxic for T cells and reactive with single-stranded DNA. They are currently studying crosses of NZB with various other strains to determine the mode of inheritance of these abnormal traits.

3. Hormones and X-linked immune response genes. These studies were performed to test the previously advanced hypothesis that the antibody response to ssDNA is controlled by an X-linked immune response response gene. The antibody response to single-stranded DNA (ssDNA) complexed to MBSA was studied in NZB, DBA/2, F_1 reciprocal hybrids, and backcross mice. It was found that the anti-ssDNA response was not linked to genes located on the X chromosome, but rather that anti-ssDNA production was regulated by sex hormones. Reciprocal F_1 hybrid males with either an NZB or a DBA/2 X chromosome were low responders; however, either castration or castration plus estrogen implants significantly increased the amount of antibody produced. Testosterone implants were immunosuppressive in both females and males. Backcross studies suggested that the ability to respond to ssDNA is inherited as an autosomal dominant trait that is further regulated by sex hormones.

Control studies were performed in hybrids of the CBA/N strain, which has a well established X-linked defect in the ability to respond to a variety of thymic independent antigens including polyriboinosinic·polyribocytidylic acid (rI·rC). F_1 hybrid males that received the CBA/N X chromosome were low-responders to rI·rC but testosterone implants in females decreased the amount of antibody produced.

These studies indicate that it is possible to separate sex hormone effects from X-linked genetic effects on immune responses. It was found that the response to ssDNA is controlled by sex hormones but not an X-linked immune response gene.

4. Cytogenetics of hemopoetic disorders. The work with Dr. Germain (Univ. Claude Bernard, Lyon, France), using the sister chromatid exchange (S.C.E.) frequencies as a criterion to compare chromosomal alterations found in (1) constitutional hemopathies associated with leukemia or preleukemia, and (2) abnormalities of the hematopoetic system induced by leukemogenic substances such as benzene and related polycyclic hydrocarbons with normal, healthy subjects as controls is being continued.

5. Experiments on pluripotential and committed hemopoietic stem cells. In collaboration with Dr. G. Brecher, Dept. of Lab. Med., School of Medicine, University of California, San Francisco, various parameters controlling the seeding and proliferation of hemopoietic stem cells when recipient animals are given isogeneic marrow transplants were studied.

Earlier studies from this group indicated that pluripotential stem cells, which are known to be normally not in the proliferative cycle, may be stimulated to divide and colonize when mice are irradiated with torsos shielded 2 hrs following a transfusion of 4×10^7 marrow cells from isogeneic donors. When irradiation was delayed for 24 hrs, only small numbers of donor cells was found and none was present in the recipients if irradiation was delayed for 4 days. These findings indicated that the noncycling stem cells needed the proliferative stimulus provided by partial body irradiation to trigger the transfused cells surviving in the shielded areas into proliferation and permanent colonization.

Further studies indicated that triggering donor stem cells into cycle is not sufficient to establish their progeny in a normal recipient and suggested that proliferative sites or niches in the recipient's spleen or marrow must be emptied of endogenous stem cells before proliferation of transfused stem cells can take place. This concept of a finite number of proliferative sites was examined in another series of experiments using hematologically recovered radiation chimeras. These animals were given whole body irradiaion and restored with transplants of isogeneic marrow. After 1 to 4 months, when marrow and peripheral blood cell counts appeared to be normal, the recipients were given a second isogeneic marrow transplant without re-irradiation. The Y chromosome, identified after C banding, was used to assess the percentage of donor cells in the recipient 2-4 weeks after the second transplant. Donor cells represented up to 25% of dividing cells in the marrow. The only exceptions were male mice restored with male marrow and subsequently given isogeneic female cells. The percentage of donor cells in that combination was less than 2%, comparable to unirradiated controls.

The proliferative site concept was further examined by treating recipient mice with ^{55}Fe prior to isogeneic marrow transplant. Pluripotent stem cells are drastically reduced after ^{55}Fe treatment while bone marrow cellularity is much less affected. This suggested that while endogenous stem cells would

still function as providers of differentiated cells, transfused stem cells might have a chance to settle and proliferate if the proliferative site or niche concept were valid. When such animals were transfused with isogeneic marrow, 5% of mitoses were of donor origin in mice that had received 1.0 mC of ^{59}Fe 12 weeks earlier and whose CFU-s were now 3%. No donor mitoses were found in animals that had received only 0.5 mC 12 weeks earlier and whose CFU-s were now 3%. No donor mitoses were found in animals that had received only 0.5 mC 12 weeks earlier and whose CFU-s were at 20%. No takes were observed in control animals given cold iron. These experiments need to be repeated within the first few days after ^{59}Fe administration when CFU-s are already low, but before a variety of compensatory events can accumulate or possibly destroy proliferative sites as well as deplete CFU-s.

6. Fluorescent in situ hybridization for gene mapping. This study is a collaborative project with Dr. P. Tischler, Harvard Medical School, Boston and Dr. B. Forget, Yale Medical School, New Haven. Chromosomes which have been identified are then hybridized to the messenger RNA and to fluorescent spheres. They are then re-examined by fluorescent microscopy, and the chromosomal location of the fluorescent spheres are noted. It is antibipated that the spheres will detect V-globin messenger RNA bound to chromosome 7 and ß-globin messenger RNA bound to chromosome 11.

7. Clinical cytogenetic studies of human congenital and developmental disorders. Patients referred to this laboratory for karyotypic diagnosis are those with various unusual genetic disorders, abnormalities in gonadal development or function, and selected cases of infertility in the male. In these of cases, minor variants or abnormalities as well as gross morphological changes are examined. With routine karyotyping many of these anomalies could have been overlooked or misinterpreted; therefore, all preparations from such patient referrals are Giemsa- and quinacrine-banded. Additional procedures such as centromeric-banding, sister chromatid exchange, and/or later replication studies are used if a chromosome abnormality is found or suspected. With the addition of new and improvement of our older, more standard banding procedures, they are now better prepared to detect karyotypic variations and more capable of correctly interpreting them.

8. Murine cytogenetic studies. In the autoimmune murine NZB strain there is development of hyperdiploidy in the spleen. In crosses of the NZB strain with the normal DBA/2 strain, hyperdiploidy was inherited as a recessive trait. Current work is underway with various other crosses to determine the effect of genetic background on the development of hyperdiploidy.

The following studies were carried out last year by the Section on Biophysical Histology (Dr. Ned Feder and Mr. Walter Stewart).

1. Studies of neuron structure and physiology with Lucifer Yellow. Further work has been done with Lucifer Yellow, an intensely fluorescent dye devised in this section for intracellular marking of individual nerve cells.

A new method for observing the same dye-injected neuron by both light and electron microscopy has shown some unexpected features of neuron structure -

for example, small, sharply delineated regions of peripheral cytoplasm which appear to be leaky to dye.

The method starts with injection of a tissue-reactive fluorescent dye, Lucifer Yellow CH, into cells that were then fixed and embedded for electron microscopy. Ultrathin ("silver") sections mounted on standard grids were examined and photographed by fluorescence microscopy. The injected neuron was readily distinguished by its intense yellow-green fluorescence, even in sections 700 A thick or less. When the same section was examined with the electron microscope, a close match, by shape and position, could be made between the cell in the electron micrograph and the same cell in the fluorescence photomicrograph.

2. Genetics of nerve cell shape. Studies have continued on the genetics of nerve cell shape, using as an experimental animal the snail Biomphalaria glabrata. B. glabrata was chosen because of its suitability for neurophysiology neuroanatomy, genetics, and convenient rearing in the laboratory. Preliminary findings are as follows: (a) over 100 presumably isogenic lines of B. glabrata have been produced by inbreeding; (b) gross anatomical and pigmentation variants have been identified, and seven were found probably to be controlled by alleles at a single locus; (c) identified neurons are being studied by several methods: injection with Lucifer Yellow, electrophsiology, histochemistry (including neurosecretion), and electron microscopy; and (d) several anatomical variants of the nervous system have been recognized, and studies of the anatomy, neurophysiology, and genetics of these variants are in progress. No significant findings have as yet been made.

The following studies have been conducted in the Section on Cellular Function and Ultrastructure during the past year (Dr. Lois Tice and staff).

1. Tight junctions in epithelial cells of the gut. The changes in tight junctions which are correlated with cell division in crypt cells of the rat duodenum have been examined using freeze-fracture techniques. In the electron microscope these mitotically active cells (mitotic index, ca. 4%) present tight junctions which are remarkably pleomorphic in character, unlike the postmitotic cells of the intestinal villus, derived from the crypt cells, which have uniform tight junction configurations. Irregular configurations, basal extensions of sealing elements, discrete elements separate from the rest of the meshwork, junctions composed primarily of particle chains rather than continuous elements, and complete gaps with in the meshwork up to 3-4 microns laterally all are present on duodenal crypt cells.

When mitosis is suppressed by administration of cytosine arabinoside, which in low doses reversibly inhibits DNA synthesis, all these anomalous junctional configurations rapidly disappear (90 to 180 min) and the crypt cell junctions come to resemble those of the intestinal villus. With recovery from the drug and further mitotic synchronization with Colcemid the cells undergo a burst of mitotic activity, with mitotic indices of up to 35%. Under these circumstances, the anomalous junctional configurations observed in normal animals are seen with increasing frequency.

Lateral gaps in the tight junction meshwork probably occur just after cytokinesis when the new daughter cells lie next to each other, and are probably bridged by particle chains which then fuse to form the continuous elements of the normal tight junction. They are of particular interest because they represent pathways for movement of antigenically active macromolecules across the epithelium, and for this reason may play a role in the genesis of some immune disease states.

2. Fine structure of the skin glands of toxin-producing frogs: The fine structure of frog toxin-producing skin glands were studied. These animals, members of the family Dendrobatidae, include a number of different species found in Central and South America. Although glands from different species exhibit minor ultrastructural differences, corresponding apparently to chemical differences in the toxins they produce, the glands all appear to conform to the same basic morphological plan.

These toxin-producing glands are found lying just beneath the epidermis of the frog, at the same level as its pigmented melanocytes. They appear to have the form of long tubules, surrounded by a discontinuous layer of smooth muscle. Within the muscle layer, the gland consists of a syncytium, with multiple nuclei at the outside. Adjacent to the nuclei is most of the cytoplasmic machinery of the cell; ER, mitochondria, Golgi apparatus etc. The interior of the gland is filled with a relatively amorphous ground substance which surrounds large, (5-6μ) poorly defined granules whose vesicular contents vary in size and electron density. These are presumed to contain the toxin.

When the frog is stimulated to secrete, the smooth muscle layer apparently contracts to extrude the inner cytoplasmic mass together with its granules (a form of apocrine secretion). Regeneration then takes place by a mechanism as yet poorly understood. At present we believe that this probably involves the aggregation of flat membranes, possibly derived from the Golgi apparatus, to form the outer vesicle shell, and subsequent insertion, by a mechanism as yet unknown, of the smaller vesicles into the outer shell. This process if currently under investigation (with Dr. Maria Neuwirth).

Dr. Charles McCutchen also of the Section on Cellular Function and Ultrastructure has carried out the following investigations this year.

1. Earlids: The active hearing protector project mentioned in the last report, has led to prototype ear defenders that permit one to stand next to an operating jackhammer without finding the noise uncomfortably loud; once the jackhammer has stopped it is possible to converse comfortably with its operator without removing the earlids or adjusting them in any way. Attempts to get earlids into use have so far failed.

2. Automatic stability of aircraft. Designers of aircraft propose automatic operation of an aircraft's controls to enable its center of gravity to be shifted rearward with a resulting gain in efficiency. They seem unaware that this can introduce a danger of tailplane stall and a consequent uncontrollable plunge. The problem can be solved by shifting the tail to the front, because then the run-away is stopped by reducing the tail's lift, which is always

possible, rather than increasing it, which may stall the tail. Attempts proceed to bring this to the attention of designers.

3. Roughness of cartilage surface. Methods have been developed to measure the roughness of cartilage surface with an ordinary microscope using vertical illumination without expensive attachments. In one, the curvature of the hollows and humps is measured by recording how far above and below the cartilage surface are the real and virtual foci formed when parallel light is reflected from the surface. Combined with the lateral distance between humps this gives an estimate of the height from the bottoms of the hollows to the tops of the humps. In another method the slope of the hump flanks in measured by observing where the reflected light passes through the back focal plane of the microscope objective. Lastly, the slopes can be shadowed and measured using a Schlieren knife edge at the exit pupil of either the microscope objective of eyepiece. The measurement is done by seeing how much change in the angle of the incident light is needed to mark the shadow boundary from one flank of each hump to the other flank, i.e. to change the Schlieren picture from all dark to all light.

Studies in the Section on Chemical Pathology by Dr. Robert Friedman and staff are concerned with various aspects of virus-cell interactions. The following projects have been carried out under the direction of Dr. Barrie Carter of the Section on Chemical Pathology.

1. Adeno-associated virus interference. Adeno-associated virus (AAV) grown in KB cells with a helper adenovirus, produced several classes of particles defined by their buoyant density in CsCl. The predominant density classes were referred to as AAV(1.45), AAV(1.41), AAV(1.35), and AAV(1.32), respectively, where the density of the particle is written in the parentheses. The AAV(1.45) and AAV(1.41) particles which contained standard genomes were the only infectious AAV particles. These infectious AAV particles exhibited autointerference. The light-density AAV(1.35) and (1.32) particles contained aberrant (deleted and/or snap-back) genomes. The light-density AAV particles were noninfectious but interfered with the replication of AAV(1.41). The interference was mediated intracellularly and resulted in inhibition of synthesis of standard (14.5 S) AAV genomes. In some cases, there was concomitantly an increased synthesis of aberrant, shorter AAV DNA. The inhibitory activity of the light-density particles was abolished by uv irradiation. These results show that the population of light AAV particles contained DI particles. The observed autointerference of AAV(1.45) or AAV(1.41) virus is postulated to be due to AAV DI particles. Replication of AAV DI genomes appeared to require the presence of replicating, standard AAV genomes. This is interpreted to mean that progeny strand replication of AAV requires an AA-specified product, presumably the AAV capsid protein. In contrast to standard, infectious AAV, the AAV DI particles alone do not inhibit replication of the helper adenovirus. The structure of the AAV DI genome has important implications for packaging of viral DNA.

2. AAV DNA replication in vitro. A complex, which is active for in vitro synthesis of adeno-associated virus (AAV) DNA, was solubilized from Vero cells that were co-infected with AAV and either adenovirus (Ad5) or a herpes simplex

virus type 1 (HSV-1) as the helper virus. The complexes from the Ad5 and HSV-1-infected cells sedimented at 23 S and 28 S, respectively. The optimal conditions for in vitro DNA synthesis for the two types of complex using the endogenous AAV template and the endogenous DNA polymerase, differed with respect to the effect of KCl and K_2SO_4 concentration. In addition the complex from HSV-1-infected cells, but not that from Ad5-infected cells, was inhibited by phosphonoacetic acid. Thus, the two complexes appear to contain different DNA polymerase activities. This was verified by phosphocellulose chromatography of the DNA polymerases solubilized from the isolated complexes. The major activity in the complex from HSV-1 infected cells was the HSV-induced DNA polymerase with lesser amounts of cellular DNA polymerase α and γ or both. The complex from the Ad5-infected cells contained mainly a cellular DNA polymerase γ.

This work and other studies suggest AAV2 DNA replication may share a common feature in a requirement for a DNA polymerase γ. Also, both Ad5 and AAV2 probably replicate via a single-strand displacement mechanism. Mitochondrial DNA also replicates by a single strand (D-loop) displacement mechanism and the mitochondria DNA polymerase is also a polymerase γ. This suggests the interesting speculation that DNA polymerase γ may be involved in strand displacement replication mechanisms.

3. AAV RNA transcription. Previous studies, which showed that stable AAV RNA synthesized in vivo anneals only to the DNA minus strand between 0.18 and 0.88 map units, may not have detected unstable or nascent RNA. Therefore, we developed a method of RNA preparation that would be expected to enrich for precursor RNA sequences. Whole cells were lysed with detergent and high salt and separated into supernatant and pellet (crude chromatin) fractions. These fractions were then separately deproteinized by proteolytic digestion and phenol extractions. DNA was removed from the preparation by two cycles of pancreatic DNase digestion and phenol extraction. Hybridization analyses of the RNA obtained from AAV/adenovirus-infected KB (human) cells revealed some AAV-specific RNA sequences that were not present in the mature 20S mRNA. These additional sequences were contained in AAV RNA molecules present in the pellet fraction, whereas the 20S AAV mRNA accumulated in the supernatant. A species of AAV-specific RNA (about 22S), which was associated only with the pellet fraction and was labeled only after a short pulse, appeared to have a kinetic relationship with the more stable cytoplasmic 20S mRNA. These putative AAV mRNA "precursors" and precursor sequences were not observed previously when conventional methods were used to obtain RNA from either whole cells or isolated nuclei.

The cytoplasmic species of AAV RNA have been mapped by analysing RNA-DNA heterduplexes either by electron microscopy or by the nuclear digestion procedure of Berk and Sharp. This work revealed 4 species of AAV mRNA containing the same spliced out region but differing in the length of the 5' leader sequence.

These studies establish the parvoviruses as an excellent system, because of their simplicity, with which to attempt to obtain correct initiation and/or post-transcriptional processing of RNA in in vitro systems.

The following studies of the Section on Chemical Pathology were carried out under Dr. Robert Friedman's supervision.

1. Control of virus genetic function in interferon-treated cells. Interferon treatment of cells producing murine leukemia viruses (MLV) or mouse mammary tumor virus (MMTV) resulted in marked decrease in virus yields. In some system this appeared to be due to an inhibition in virus budding from the plasma membrane of the cell; in others, virus with marked decrease in infectivity was produced. Several proteins of MLV seem to be altered in virus derived from interferon-treated cells. In addition, in an RNA virus from an entirely different group, vesicular stomatitis virus (VSV), particles of low infectivity are produced after interferon treatment. In this system, there appears to be an inhibition in the incorporation of the viral glycoprotein into the virion. These changes may be related to interferon induced alterations in the cell plasma membrane.

In addition, the mechanism of interferon-induced inhibition of virus protein synthesis in cell-free systems is being studied.

2. Persistent virus infection in tissue culture. The prolonged infection of a normally lytic virus, vesicular stomatitis virus (VSV) in mouse L cells was initiated in a novel way by interferon treatment of the cells before virus challenge. The mechanism of maintenance of this persistent infection was due to several factors. The major factors were the development of temperature-sensitive small plaque mutants and the production of endogenous interferon. No evidence for defective interfering VSV particles was uncovered. The L cells employed in these studies were also unable to amplify exogenously produced DI particles of VSV.

The following studies have been carried out under the supervision of Dr. Francis Jay of the Section on Chemical Pathology.

1. Studies on SV40 early antigens. In studies on SV40 early antigens, $Ad2^{+}ND2$ induced in infected cells two SV40 specific peptides (42,000 + 56,000 in molecular weight). $Ad2^{+}ND1$ induced only a 28,000 in molecular weight peptide. The $Ad2^{+}ND1$ olgment is sufficient to induce SV40-specific tumor antigen in mice.

2. Studies on AAV proteins. With 2-dimentronal gel electrophoresis a 4th AAV structural protein was detected. Each of the 4 viral proteins contains multiple isoelectric species. VP1, 2 + 3 share many tryptic peptides. VP1 + 3 also contain unique peptides.

In addition, the Office of the Chief, under the supervision of Dr. Friedman handles animal and human tissues from the whole institute. This year over 4,000 slides were prepared from this material. Much of this involved specialized histopathologic preparations (Miss V. Reveal).

The Office of the Chief also carried out collaborative studies on the electron microscope with many NIAMDD investigators (Dr. H. Bladen) as a service to the institute.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,001-25 LEP

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (30 characters or less)

Preparation of stained tissue sections for investigation and diagnostic purposes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Virginia Reveal Head, Tissue Preparation Lab. LEP NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIAMDD, LEP, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The tissue laboratory will continue to provide technical assistance to the investigators of the Laboratory of Experimental Pathology and to those of other units at the NIH who require technical help. A considerable part of the tissue laboratory's work is concerned with providing a wide variety of special histologic techniques adjusted to the various research projects as shown below.

	Specimen Accessioned	Stained Slides Routine	Stained Slides Special	Spare Slides	Total
Animals	407	566	1635	2672	
Human	9	9		9	
	416	575	1635	2681	
		2210			
					4891

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 21,002-14 LEP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cellular Function and Ultrastructure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lois W. Tice	Chief, Section on Cellular Function and Ultrastructure	LEP	NIAMDD
OTHER:	Maria Neuwirth	Visiting Fellow	LEP	NIAMDD
	Raymond Carter	Biomedical Technician	LEP	NIAMDD
	Marjorie Cahill	Bio. Lab. Tech.	LEP	NIAMDD

COOPERATING UNITS (if any)

Dr. J. W. Daly, LC:NIAMDD
Dr. C. R. Creveling; LC:NIAMDD

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Cellular Function and Ultrastructure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Changes in tight junctions of rat duodenal crypt cells were correlated with changes in mitotic activity.

PHS-6040
(Rev. 10-76)

Project description:

Epithelial cells in many tissues are arranged in a roughly hexagonal array, linked together at their apical ends by tight junctions, narrow belts of sealing elements which fuse their plasma membranes together. In freeze-fracture replicas these fusions are represented by an anastomosing network of ridges and furrows. These sealing belts appear to regulate transepithelial ionic resistance and, since they are impermeable to macromolecules, to maintain an immunological competent barrier across the epithelium. In the gut, for example, the tight junctions between epithelial cells prevent gut contents from penetrating between the cells to reach the extracellular space. Similarly, in the thyroid gland, tight junctions restrict thyroglobulin to the interior of the thyroid follicle.

When an epithelial cell divides, it introduces a new unit into the epithelial cell packing. The result of this event is that daughter cells must synthesize new junctional material and rearrange their prexisting junctions to maintain or restore the junctional seals around them. In addition, other cells in the epithelium move with respect to one another with the result that their junctional material must also be rearranged.

We have examined the changes in tight junctions which are correlated with cell division in crypt cells of the rat duodenum using freeze-fracture techniques. In the electron microscope these mitotically active cells (mitotic index, ca. 4%) present tight junctions which are remarkably pleomorphic in character, unlike the postmitotic cells of the intestinal villus, derived from the crypt cells, which have uniform tight junction configurations. Irregular configurations, basal extensions of sealing elements, discrete elements separate from the rest of the meshwork, junctions composed primarily of particle chains rather than continuous elements, and complete gaps with in the meshwork up to 3-4 microns laterally, all are present on duodenal crypt cells.

When mitosis is suppressed by administration of cytosine arabinoside, which in low doses reversibly inhibits DNA synthesis, all these anomalous junctional configurations rapidly disappear (90 to 180 min) and the crypt cell junctions come to resemble those of the intestinal villus. With recovery from the drug and further mitotic synchronization with Colcemid the cells undergo a burst of mitotic activity, with mitotic indices of up to 35%. Under these circumstances, the anomalous junctional configureations observed in normal animals are seen with increasing frequency.

Lateral gaps in the tight junction meshwork probably occur just after cytokinesis when the new daughter cells lie next to each other, and are probably bridged by particle chains which the fuse to form the continuous elements of the normal tight junction. They are of particular interest because they represent pathways for movement of antigenically active macromolecules across the epithelium, and for this reason may play a role in the genesis of some immune disease states.

Publication:

Neuwirth, M., Daly, J.W., Myers, C.W. and Tice, L.W.: Morphology of the granular secretory glands in skin of poison-dart frogs. Tissue & Cell 1979 in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,003-11 LEP

PERIOD COVERED

October 1, 1978 through September 30,1979

TITLE OF PROJECT (80 characters or less)

Biomechanics and Related Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. W. McCutchen Research Physicist LEP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Section on Cellular Function and Ultrastructure.

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/2	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Earlids, the active hearing protectors have been developed to the prototype stage.

Automatic stability is being proposed by designers to improve the efficiency of aircraft. I point out that this can make the aircraft liable to divergent tailplane stall, and that the danger can be eliminated by putting the tail in front.

Methods have been developed to measure the roughness of cartilage surface with an ordinary microscope using vertical illumination and without expensive attachments.

PHS-6040
(Rev. 10-76)

Project description:

Earlids:

The active hearing protector project mentioned in the last report has led to prototype ear defenders that permit one to stand next to an operating jackhammer without finding the noise uncomfortably loud, and then, once the jackhammer has stopped, to converse comfortably with its operator without removing the earlids or adjusting them in any way. Attempts to get earlids into use have so far failed.

Automatic Stability of Aircraft:

Designers of aircraft propose automatic operation of an aircraft's controls to enable its center of gravity to be shifted rearward with a resulting gain in efficiency. They seem unaware that this can introduce a danger of tail-plane stall and a consequent uncontrollable rear or plunge. The problem can be solved by shifting the tail to the front, because then the run-away is stopped by reducing the tails lift, which is always possible, rather than increasing it, which may stall the tail. Attempts proceed to bring this to the attention of designers.

Roughness of Cartilage Surface:

Methods have been developed to measure the roughness of cartilage surface with an ordinary microscope using vertical illumination without expensive attachments. In one, the curvature of the hollows and humps is measured by recording how far above and below the cartilage surface are the real and vertual foci formed when parallel light is reflected from the surface. Combined with the lateral distance between humps this gives an estimate of the height from the bottoms of the hollows to the tops of the humps. In another method the slope of the hump flanks in measured by observing where the reflected light passes through the back focal plane of the microscope objective. Lastly, the slopes can be shadowed and measured using a schlierin knife edge at the exit pupil of either the microscope objective or eyepiece. The measurement is done by seeing how much change in the angle of the incident light is needed to march the shadow boundary from one flank of each hump to the other flank, i.e. to change the schlieren picture from all dark to all light.

Publications:

McCutchen, C. W.: Comment on "A model for the distribution of HAP crystallites in bone - an hypothesis" by S. Lees. Calcif. Tiss. Int. 27: 53-56, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 21,004-06 LEP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Possible Mechanisms of Persistence of Vesicular Stomatitis Virus in Mouse L Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert M. Friedman	Chief, Laboratory of Experimental Pathology	LEP	NIAMDD
OTHER:	Janet M. Ramseur	Research Biologist	LBV	NIAID

COOPERATING UNITS (if any)

NIAID

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Chemical Pathology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2-1/3	1-1/3	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The prolonged infection of a normally lytic virus, vesicular stomatitis virus (VSV) in mouse L cells was initiated in a novel way by interferon treatment of the cells before virus challenge. The mechanism of maintenance of this persistent infection was due to several factors. The major factors were the development of temperature-sensitive small plaque mutants and the production of endogenous interferon. No evidence for defective interfering VSV particles was uncovered. The L cells employed in these studies were also unable to amplify exogenously produced DI particles of VSV.

2-C-6040
(Rev. 10-76)

Project description:

Ly cells were treated with 100 reference units/ml of mouse interferon and then infected with a wild type vesicular stomatitis virus (VSV) at a multiplicities of 0.1-60 virus plaque forming units per cell, prolonged infection of cultures ensued, lasting from 14 to at least 60 days. Less than 1% of the cells produced infectious virus, but more than 10% produced detectable levels of VSV antigens. The prolonged infection could not be established, if the cell type was changed to VERO or AKR; if Sindbis was employed; or, if the cells were grown at 32°C rather than at 37°C.

Temperature sensitive mutants which evolved and dominated the persistent infection contributed to the maintenance of the prolonged infection. The virus produced during the prolonged infections grew to lower titers at 39°C than at 32°C and the plaque size at 39°C progressively decreased with the length of time the infection persisted. The plaque size was also smaller than wild type VSV at 37°C; but, at 32°C the virus from the prolonged infection and wild type VSV grew to a similar plaque size. Lowering the incubation temperature of the cultures at day 5 post infection from 37°C to 32°C resulted in an increase in virus production and in virus-induced cytopathology. After cloning the virus from the chronic infection, the virus plaque size remained small at 39°C and 37°C and also the virus appeared to be heat labile at 41°C. Growth curves of these mutants were distinct from wild type VSV at 39°C and 32°C.

Results indicated endogenous interferon to be another factor in the maintenance of the VSV-L cell prolonged infection. Indirect evidence for the production of interferon was supplied by the resistance to superinfection of prolonged L cell-VSV infections with wild type VSV or with a heterologous virus, encephalomyocarditis virus. In addition, low concentrations of endogenous interferon were detected in the supernatant fluids of chronically infected cells by plaque reduction assays. More direct evidence for endogenous interferon production was the marked increase in virus titers and the termination of the chronic infection by treatment of the prolonged L cell infection with rabbit anti-mouse interferon globulin. This effect was not initiated by rabbit anti-human interferon globulin.

No defective interfering (DI) particles were detected in the chronically infected VSV-Ly cell cultures; therefore, it was unlikely that DI particles were factors in the maintenance of this persistent infection. After 4 passages in Ly cells at high multiplicities of VSV from a prolonged infection there was no inhibition of virus growth and virus from the prolonged infection had the same distribution of intracellular and extracellular RNA forms as was found in wild-type virus passed four times in Ly cells. Also, the intracellular and extracellular RNA forms of virus taken directly from a prolonged infection of Ly cells were indistinguishable from those of the wild-type virus. A morphological examination of VSV from a prolonged infection did not show a significant

number of small, defective viral forms after four passages in Ly cells at a high multiplicity of infection.

Ly cells appeared to be poor producers of DI particles. Wild type plaque purified virus passed three times at high multiplicities in L cells failed to decrease infectious virus titer. Extracellular VSV RNA patterns only showed virion RNA of 42S. When DI particles formed in BHK cells after high multiplicity passage were grown in the presence of wild type plaque purified VSV in L cells, no interference with normal infectious virus replication was observed and only 42S RNA was found in virions. Therefore, replication of DI particles in L cells is defective.

Publications:

Friedman, R.M. and Ramseur, J. M.: Mechanisms of persistent infections by cytopathic viruses in tissue culture. Arch. Vriol. 60: 83-103, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,005-12 LEP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Histochemistry: Principles, Methods & Applications

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ned Feder	Chief, Section on Biophysical Histology	LEP	NIAMDD
OTHER:	Walter Stewart	Senior Staff Fellow	LEP	NIAMDD

COOPERATING UNITS (if any)
Dr. Raymond F. Cheri (NHLBI)
Dr. Steven Wise (NIMH)

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Biophysical Histology

INSTITUTE AND LOCATION
NIAMDD:NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	2	3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continuing studies with the dye, Lucifer Yellow, have been carried out in conjunction with electron microscopy.

Studies have continued on the genetics nerve cell shape using the snail Biomphalaria glabrata as an experimental animal.

PHS-6040
(Rev. 10-76)

Project description: Z01 AM 21,005-12 LEP

Studies of neuron structure and physiology with Lucifer Yellow

Further work has been done with Lucifer Yellow, an intensely fluorescent dye devised in this laboratory for intracellular marking of individual nerve cells.

A new method for observing the same dye-injected neuron by both light and electron microscopy has shown some unexpected features of neuron structure - for example, small, sharply delineated regions of peripheral cytoplasm which appear to be leaky to dye.

The method starts with injection of a tissue-reactive fluorescent dye, Lucifer Yellow CH, into cells that were then fixed and embedded for electron microscopy. Ultrathin ("silver") sections mounted on standard grids were examined and photographed by fluorescence microscopy. The injected neuron was readily distinguished by its intense yellow-green fluorescence, even in sections 700 A thick or less. When the same section was examined with the electron microscope, a close match, by shape and position, could be made between the cell in the electron micrograph and the same cell in the fluorescence photomicrograph.

Genetics of nerve cell shape

Studies have continued on the genetics of nerve cell shape, using as an experimental animal the snail Biomphalaria glabrata. B. glabrata was chosen because of its suitability for neurophysiology, neuroanatomy, genetics, and convenient rearing in the laboratory. Our preliminary findings are as follows. (a) Over 100 presumably isogenic lines of B. glabrata have been produced by inbreeding. (b) Gross anatomical and pigmentation variants have been identified, and seven were found probably to be controlled by alleles at a single locus. (c) Identified neurons are being studied by several methods: injection with Lucifer Yellow, electrophysiology, histochemistry (including neurosecretion), and electron microscopy. (d) Several anatomical variants of the nervous system have been recognized, and studies of the anatomy, neurophysiology, and genetics of these variants are in progress. No significant findings have been made.

Publication:

Stewart, W. W.: Functional connections between cells as revealed by dye-coupling with a highly fluorescent naphthalimide tracers. Cell, 14: 741-759, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21006-11 LEP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Protein and Proteolytic Enzyme Interrelationships in Normal and Disease States.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George G. Glenner	Chief, Section on Molecular Pathology	LEP	NIAMDD
OTHER:	Shegirishi Fujihara	Visiting Fellow	LEP	NIAMDD
	Katsuikho Fukasawa	Visiting Fellow	LEP	NIAMDD

COOPERATING UNITS (if any)
Dr. W. K. Engel, N:MN
Dr. K. Weisgraber, H:IR:OD
Dr. M. Rodriques, EI:CB
Dr. P.P. Costa, Instituto de Neurologia, Porto, Portugal

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Molecular Pathology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bldg. 10, Rm. 3N-112, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3	2

CHECK APPROPRIATE BOX(ES)
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to study the chemistry and structure of amyloid and amyloid related proteins. The main topics of present interest are:

1) Isolation and chemical characterization of amyloid fibril protein from various types of systemic amyloidosis and localized amyloid deposits.

2) Mechanism of formation of amyloid deposits from serum precursors.

3) Isolation and purification of proteolytic enzymes involved in amyloid fibril formation.

4) Immunochemical characterization of amyloid fibrils from different syndromes in tissue section for diagnostic and therapeutic purposes.

PHS-6040
(Rev. 10-76)

Origin and Nature of Human, A myloid Fibril Proteins and Precursors.

Composition of Amyloid Fibrils From a Patient with Myeloma:

A large group of amyloid fibril proteins have been shown to possess as their major protein component some portion of an immunoglobulin light polypeptide chain. Acrylamide-gel electrophoresis of purified amyloid fibril protein (LAm) obtained from a patient with myeloma and amyloidosis demonstrates three major protein components which are shown to share certain structural properties with a lambda-type Bence Jones (LBJ) light chain isolated from the patient's urine. The evidence linking the amyloid components and urinary light chain is as follows: (1) Peptide maps are nearly identical for both proteins. We attribute minor differences in the maps to variations in the carboxy-terminal tryptic peptides obtained from the two smaller amyloid protein components and not to the presence of a nonimmunoglobulin molecule. (2) The amino termini of both proteins were unreactive to DNFB. The amino acid compositions of the N-terminal chymotryptic tetrapeptides obtained from the two proteins are identical to each other and the amino-terminal chymotryptic tetrapeptides of lambda-type light chains of subgroup V_{II} or V_{IV}. (3) Immunochemical studies demonstrate shared idiotypic determinants between the two proteins. Immunochemical analysis further demonstrates the presence in the patient's serum of a protein having idiotypic determinants in common with both LBJ and LAm. These results confirm the characterization of LAm as a lambda-type amyloid fibril protein exhibiting common structural determinants with the Bence Jones protein that was present in the patient's serum and urine.

In most instances of immunoglobulin-related amyloid, the protein component of the amyloid is homogeneous and consists of either an intact light polypeptide chain or an amino-terminal fragment of a light chain. The demonstration by acrylamide-gel electrophoresis after reduction and alkylation of three major protein components, all with antigenic determinants that are comparable to those of the Bence Jones protein, suggests the presence in this particular case of multiple amino-terminal light chain fragments. Molecular weight determination of the three major components of LAm reveals values of 23,000, 17,500, and 12,500, consistent with those expected for an intact light immunoglobulin chain, an amino-terminal fragment slightly larger than a light chain variable region and an amino-terminal fragment of intermediate size. The finding on peptide maps of two peptides unique to the isolated amyloid is compatible with this suggestion. These results are consistent with the conclusion that such cases of amyloidosis as the above are the result of limited proteolysis of an intact light chain with fibril formation involving the N-terminal variable region as suggested previously (Glenner, G.G. et al. Semin. Hematol. 10, 65 (1973).

Pathogenesis of Cerebral Amyloid Fibrils and Presenile Dementia: The most likely mechanism for the pathologic changes seen in the majority of cases of Alzheimer's syndrome (presenile dementia) that is consistent with the

morphologic, histochemical, immunohistochemical and chemical data reported from this and other laboratories and other mechanisms potentially leading to the formation of neural degeneration, neuritic plaques and paired helical filaments synonymous with presenile dementia is briefly summarized here. This theory assumes that the pathological process initiates in the brain by means of the proteolytic activity of the unique tight junction, cerebral endothelial cells and/or during vesicular transport by pericytes acting on either a normal or abnormal protein (e. g. a monoclonal immunoglobulin protein, most likely a Fab fragment, or other "amyloidogenic" serum protein) to form amyloid fibrils by lysosomal catheptic activity. When this involves intracortical vessels, particularly those of capillary and precapillary type, a break in the blood-brain barrier occurs, permitting a chronic influx of serum proteins throughout the neuropil. One or more of these serum proteins chronically and irreversibly affects the neural cells to produce eventually a granulovacular degeneration and consequent abnormalities in the extracellular aggregation and/or chemical characteristics of unassembled neurofilament protein present within neurities. Such physiochemical or chemical changes, conducive to amyloid fibril formation elsewhere, produces in the neuritic environment paired helical filaments and other pathologic neurofilamentous figures. Intraneuritic degeneration simultaneously or subsequently occurs with dense bodies, i.e. lysosomes containing degraded neurofilamentous protein, accumulating in the neuritic cytoplasm. This partially cleaved neurofilamentous protein is deposited extracellularly from degenerating neurites by a process of exocytosis and is acted upon further by the lysosomal complement of microglia (macrophages) to produce the amyloid core of the neuritic plaque. Other pathogenetic mechanisms of lesser frequency associated with neuritic plaque formation, but occurring in the absence of Congophilic angiopathy, probably also progress in the remaining cases of presenile (and senile) dementia by an ictogenic (ictus: recurring stress) extrinsic stimulus. Other mechanisms, e.g. in Down's syndrome and the genomic Alzheimer's degenerative abnormalities of familial type, may affect the neural cell at the level of DNA or RNA synthesis to afford abnormal neurofilamentous protein and/or aggregation, resulting also in neurofibrillary tangles in the absence of Congophilic angiopathy. Consequently, Alzheimer's degeneration may result from a variety of causes, the principle one being the compromise of the blood-brain barrier resulting from Congophilic microangiopathy. Congophilic angiopathy in the absence of neuritic plaques is on the other hand associated with macroangiopathy, i.e. involvement of the leptomeningeal or extra-cortical pial vessels where amyloid deposits only infrequently infiltrate the Virchow-Robin space to compromise the blood-brain barrier. The differences in localization between Congophilic microangiopathic changes and sites of Alzheimer's neurofibrillary degeneration previously reported, neither militate against or contravene the concept of a causal relationship between these processes in view of the seneral acceptance of the theory of pathoclises, e.g. in this case the neurotoxic serum substance(s) passing the blood-brain barrier and having an affinity for those specific neural systems and cell types involved in Alzheimer's degeneration. The chronicity of this effect is emphasized. In conclusion, it appears that in a large proportion of cases of presenile dementia the major causal mechanism is by compromise of the blood-brain barrier as the result of Congophilic microangiopathy to provide chronic access of serum proteins

to the cerebrum; the first implication of amyloid deposition in the pathogenesis of a subsequent disease complex.

Publication:

T. B. Orstavik and G. G. Glenner: Localization of kallikrein and its relation to other trypsin-like esterases in the rat pancreas. A Comparison with the submandibular gland. Acta. Physiol. Scand. 103: 384-393, 1978.

G. G. Glenner: Current knowledge of amyloid deposits as applied to senile plaques and Congophilic angiopathy. In Alzheimer's Disease: Senile dementia and related disorders (Aging, Vol., 7), Katzman, R., Terry, R.D., Bick, K. L. (Eds): Raven Press, N.Y. 493-502, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 21,007-08 LEP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Acute Phase Reactants and Amyloidosis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Jean D. Sipe Research Chemist LEP NIAMDD

COOPERATING UNITS (if any)

Stefanie N. Vogel and David L. Rosenstreich, Lab. of Microbiology and Immunology, NIDR; Keith P.W.J. McAdam, Dept. of Med., Tufts Univ., Boston, Mass.

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION

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INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryalnd 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.5	0.25

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Serum amyloid A (SAA) is an acute phase reactant that was detected within the past decade by its cross-reaction with antibodies raised to denatured amyloid A Fibrils. The origin and structure of SAA and its role in the pathogenesis of secondary amyloidosis has been investigated, employing the mouse as an experimental model. Induction of the acute phase SAA response by inflammatory agents such as endotoxin has thus far been observed only in vivo. We have shown that two cell populations are involved in the acute phase SAA response to lipopolysaccharide (LPS). A macrophage response to LPS gives rise to SAA inducer, which in turn stimulates SAA synthesis by liver and possibly other tissues.

Three stages in the SAA response to LPS and other agents of inflammation have been observed: a latent period of 2 to 3 hr during which the SAA concentration remains at baseline values and in which SAA inducer appears; the period of SAA synthesis which lasts for 24 hr past induction, and the period of deinduction during which SAA synthesis ceases and SAA concentration returns to baseline values by 48 hrs after induction.

PHS-6040
(Rev. 10-76)

Project description:

Mechanism of the Acute Phase SAA Response.

The mechanism by which LPS stimulates an acute phase SAA response in C3H mice has been studied. C3H/HeJ mice do not produce a significant SAA response to phenol extracted E. coli K235 LPS, whereas C3H/HeN mice exhibit a several hundred fold increase in SAA concentration at the peak of the acute phase response. We have therefore utilized this strain pair to investigate the possibility of an SAA inducer and have found that macrophages make an SAA inducer in response to LPS. This factor appears in the blood of C3H/HeN mice approximately 1 hr after administration of LPS. When passively administered, it can induce C3H/HeJ mice to produce SAA, although they are resistant to the LPS itself. SAA inducer is detectable in the culture medium of LPS treated C3H/HeN macrophages but not spleen cells. Thus, two stages in the induction of the acute phase SAA response have been defined: a latent period of 2 to 3 hr during which the SAA concentration remains at baseline values and in which SAA inducer appears, and the period of synthesis of SAA which lasts for approximately 24 hr past induction. The third part of the SAA response to LPS is deinduction, when the SAA concentration returns to baseline values.

These findings demonstrate the mechanism by which LPS produces increased SAA concentrations and lay a logical framework for explaining how chronic inflammation may result in amyloidosis.

Partial Reversal of LPS Unresponsiveness in BCG-Infected C3H/HeJ Mice.

C3H/HeJ mice exhibit a marked insensitivity to lipopolysaccharide (LPS) in vivo. Pretreatment of these mice with viable BCG organisms 11 days prior to LPS administration renders them sensitive to LPS, lowering their LPS LD_{50} from >8 mg to 280 µg. Other in vivo responses in parallel with this enhanced endotoxicity are increased in BCG infected C3H/HeJ mice. These include: 1) the production of interferon 2 hrs following the injection of 10 µg LPS i.v. (94 units of interferon in BCG infected C3H/HeJ mice compared with 21 units in controls) and 2) the production of the acute phase reactant, serum amyloid A (SAA) (485 µg/ml in BCG infected mice compared with 111 µg/ml in controls). This data suggests that the mechanisms responsible for LPS sensitivity are operative within 2 hrs of LPS administration. C3H/HeN mice normally exhibit slightly greater LPS sensitivity than BCG infected C3H/HeJ mice (LD_{50} = 76 µg), but also exhibit enhanced sensitivity to LPS following infection with BCG (LD_{50} = 9 µg). Thus, the ability of BCG to induce LPS sensitivity in C3H/HeJ mice suggests that LPS unresponsiveness is not due to an absolute defect in this strain, but rather, a partially reversible state of hyporesponsiveness. Once properly stimulated, e.g., with BCG, C3H/HeJ mice exhibit a partial reversal of LPS insensitivity, approaching the response of normal C3H/HeN mice.

Publications:

Sipe, J. D.: Detection of antibodies to the amyloid fibril protein AA by means of solid phase radioimmunoassay for AA. J. Immunol. Methods. 23: 253-258, 1978.

Sipe, J. D.: Induction of the acute phase serum protein SAA requires both RNA and protein synthesis. Brit. J. Exp. Path. 59: 305-310, 1978.

Hijmans, W. and Sipe, J. D.: Levels of the serum amyloid A Protein (SAA) in normal persons of different age groups. Clin. Exp. Immun. 35: 96-100, 1979.

Sipe, J. D., Vogel, S. N., Ryan, J. L., McAdam, K. P. W. J., and Rosenstreich, D. L.: Detection of a mediator derived from endotoxin-stimulated macrophages that induces the acute phase SAA response in mice. J. Exp. Med. (in press).

Vogel, S. N., Moore, R. N., Sipe, J. D. and Rosenstreich, D. L.: Partial reversal of LPS unresponsiveness in BCG-infected C3H/HeJ mice. Fed Proc. (in preparation).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,008-13 LEP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cytogenetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. H. Tjio	Chief, Section on Cytogenetics	LEP	NIAMDD
OTHER:	B. J. White	Medical Officer	LEP	NIAMDD
	E. S. Raveche	Research Chemist	LEP	NIAMDD

COOPERATING UNITS (if any)

U. Reinke Harvard Medical School, Boston
G. Brecher Univ. Calif. Medical Center, San Francisco
P. Tischler Harvard Medical School, Boston, A. Steinberg (AR:NIAMDD)

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Cytogenetics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Application of differential staining techniques and continued improvement of established methods are basic to all of the projects conducted in the laboratory.

2. Human cytogenetic studies of patients with congenital and developmental disorders as well as studies of mutagen-induced chromosomal abnormalities are continuing.

3. Murine cytogenetic studies include evaluation of mitotic and meiotic chromosomes and karyotypic evaluation of cells in hematopoetic tissue regeneration experiments. These are part of projects utilizing the mouse as a model for human diseases.

PHS-6040
(Rev. 10-76)

Project description:

Cytogenetics of Hemopoetic Disorders:

The work with Dr. Germain (University Claude-bernard, Lyon, France), using the sister chromatid exchange (S.C.E.) frequencies as a criterion to compare chromosomal alterations found in (1) constitutional hemopathies associated with leukemia or preleukemia, and (2) abnormalities of the hematopoetic system induced by leukemogenic substances such as benzene and related polycyclic hydrocarbons with normal, healthy subjects as controls is being continued.

Experiments on Pluripotential and Committed Hemopoietic Stem Cells:

In collaboration with Dr. G. Brecher, Department of Laboratory Medicine, School of Medicine, University of California, San Francisco, we have explored various parameters controlling the seeding and proliferation of hemopoietic stem cells when recipient animals are given isogeneic marrow transplants.

Earlier studies in our laboratories indicated that pluripotential stem cells, which are known to be normally not in the proliferative cycle, may be stimulated to divide and colonize when mice are irradiated with torsos shielded 2 hours following a transfusion of 4×10^7 marrow cells from isogeneic donors. When irradiation was delayed for 24 hours, only small numbers of donor cells were found and none were present in the recipients if irradiation was delayed for 4 days. These findings indicated that the non-cycling stem cells needed the proliferative stimulus provided by partial body irradiation to trigger the transfused cells surviving in the shielded areas into proliferation and permanent colonization.

To explore this notion further, normal recipients were given marrow from Neostigmine treated donors. Neostigmine has been shown to induce DNA synthesis in 100% of CFU-s. In the spleens of these recipient mice, 7%-32% of dividing cells were of donor origin when chromosome analyses were performed 4-8 days after transfusion. However, permanent colonization did not take place as few cells of donor origin were found in bone marrow at 4-8 days; and, at later dates, no dividing donor cells could be found in either spleen or marrow. These experiments indicated that triggering donor stemcells into cycle is not sufficient to establish their progeny in a normal recipient and suggested that proliferative sites or niches in the recipient's spleen or marrow must be emptied of endogenous stem cells before proliferation of transfused stem cells can take place.

This concept of a finite number of proliferative sites was examined in another series of experiments using hematologically recovered radiation chimeras. These animals were given whole body irradiation and restored with transplants of isogeneic marrow. After 1 to 4 months, when marrow and peripheral blood cell counts appeared to be normal, the recipients were given a second isogeneic marrow transplant without re-irradiation. The Y chromosome, identified after C banding, was used to assess the percentage of donor

cells in the recipient 2-4 weeks after the second transplant. Donor cells represented up to 25% of dividing cells in the marrow. The only exceptions were male mice restored with male marrow and subsequently given isogeneic female cells. The percentage of donor cells in that combination was less than 2%, comparable to unirradiated controls. The successful seeding by the second marrow transplant in hematologically normal mice appeared to be explainable by the observation of Vos (Cell Tissue Kinetics, 1972) that CFU-s in irradiated and restored mice remain at 85% of normal for prolonged periods, implying the existence of empty proliferative sites which might then be filled by a second marrow transplant. However, when a third isogeneic marrow transplant was administered, seeding from that transfusion also took place, although the second transplant had been expected to fill all empty proliferative sites.

In a final series of experiments, in collaboration with Dr. Reincke of Brookhaven Laboratories and with Dr. Brecher in San Francisco, the proliferative site concept was further examined by treating recipient mice with ^{55}Fe prior to isogeneic marrow transplant. Pluripotent stem cells are drastically reduced after ^{55}Fe treatment while bone marrow cellularity is much less affected. This suggested that while endogenous stem cells would still function as providers of differentiated cells, transfused stem cells might have a chan e to settle and proliferate if the proliferative site or niche concept were valid. When such animals were transfused with isogeneic marrow, 5% of mitoses were of donor origin in mice that had received 1.0 mC of ^{55}Fe 12 weeks earlier and whose CFU-s were now 3%. No donor mitoses were found in animals that had received only 0.5 mC 12 weeks earlier and whose CFU-s were at 20%. No takes were observed in control animals given cold iron. These experiments need to be repeated within the first few days after ^{55}Fe administration when CFU-s are already low, but before a variety of compensatory events can accumulate or possibly destroy proliferative sites as well as deplete CFU-s.

We plan to continue these experiments in an effort to heighten our understanding of the factors governing the proliferation of transfused stem cells in hematologically normal animals. Additional studies will include exploration of male versus female transplantibility; the effect of radiomimetic drugs versus irradiation on seeding after hematologic recovery; transplantibility examined as a local versus general phenomenon; and we shall further explore the concept of fixed numbers of proliferative sites or niches to explain variations in the success of isogeneic transplants under various conditions.

Fluorescent in Situ Hybridization for Gene Mapping:

This study is a collaborative project with Dr. P. Tischler, Harvard Medical School, Boston and Dr. B. Forget, Yale Medical School, New Haven. Chromosomes which have been identified are then hybridized to the messenger RNA and to fluorescent spheres. They are then re-examined by fluorescent microscopy, and the chromosomal location of the fluorescent spheres are noted. We anticipate that the spheres will detect α-globin messenger RNA bound to chromosome 7 and β-globin messenger RNA bound to chromosome 11.

Clinical Cytogenetic Studies of Human Congenital and Developmental Disorders:

Patients referred to our laboratory for karyotypic diagnosis are those with various unusual genetic disorders, abnormalities in gonadal development or function, and selected cases of infertility in the male. In these types of cases, we are looking for minor variants or abnormalities as well as gross morphological changes. With routine karyotyping many of these anomalies could be overlooked or misinterpreted; therefore, all preparations from such patient referrals are Giemsa- and quinacrine-banded. Additional procedures such as centromeric-banding, sister chromatid exchange, and/or later replication studies are used if a chromosome abnormality is found or suspected. With the addition of new and improvement of our older, more standard banding procedures, we are now better prepared to detect karyotypic variations and more capable of correctly interpreting them.

Murine Cytogenetic Studies:

This study is a collaborative project with Dr. Elizabeth Raveche and Dr. Alfred D. Steinberg, NIAMDD. We have found in the autoimmune murine NZB strain the development of hyperdiploidy in the spleen. In crosses of the NZB strain with the normal DBA/2 strain, hyperdiploidy was inherited as a recessive trait. Current work is underway with various other crosses to determine the effect of genetic background on the development of hyperdiploidy.

Publication:

White, B. J., Crandall, C., Raveche, E. S. and Tjio, J. H.: Laboratory mice carrying three pairs of Robertsonian translocations: Establishment of a strain and analysis of meiotic segregation. Cytogenetics and Cell Genetics. 21: 113-138, 1978.

Raveche, E. S., Tjio, J. H. and Steinberg, A. D.: Genetic studies in NZB mice II. Hyperdiploidy in the spleen of NZB mice and their hybrids. Cytogenetics and Cell Genetics. 23: 182-193, 1979.

Raveche, E. S., Tjio, J. H. and Steinberg, A. D.: Genetic studies in NZB mice III. Induced anti-nucleic acid antibody production. J. Immunology 122: 1454-1460, 1979.

Tjio, J. H.: Citation classic. Current Contents. 10: 14, 1979.

Brecher, G., Tjio, J. H., Haley, J. E., Narla, J., Beal, S. L.: Transplantation of murine bone marrow without prior host irradiation. Blood Cells (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 21,009-12 LEP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cytogenetic Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. J. White	Research Medical Officer	LEP	NIAMDD
OTHER:	T.-H. Tjio	Chief, Section on Cytogenetics	LEP	NIAMDD

COOPERATING UNITS (if any)

E. Bersu, Dept. Anatomy, University of Wisconsin, Madison; J. Goudsmit and C. Morrow, CNS, NINCDS: W. Strober, MET, NCI: C. Boone, LC, NCI.

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Cytogenetics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Cytogenetic and reproductive studies of murine Robertsonian translocation carriers. Analysis of adult and fetal somatic and gonadal tissues using chromosomal banding techniques in order to study meiotic nondisjunction and characteristics of aneuploid progeny from translocation heterozygotes.

2) Cytogenetic and dermatoglyphic analysis of patients with congenital defects, recognized dysmorphic syndromes, abnormal gonadal development and function, and other genetic disorders. Techniques used for cytogenetic studies include Giemsa-, quinacrine-, and centromeric-banding, as well as fluorescent-Giemsa analysis of chromosomal replication sequences. Cytogenetic, dermatoglyphic, and other clinical studies are correlated.

3) Karyotypic evaluation of potentially abnormal tissues. These studies utilize a variety of chromosomal banding methods for analysis of tissues from laboratory animals (primate and murine) suspected of having chromosomal abnormalities. Relationship of cytogenetic findings to genetic background, development of tumors, and reproductive fitness is emphasized.

Project description:

Cytogenetic and Reproductive Studies of Murine Robertsonian Translocation Carriers.

Our laboratory continues to maintain the following randomly-bred homozygous Robertsonian translocation strains: Rb(5.19)1Wh, Rb(9.19)163H, Rb(8.17)1M, Rb(8.19)1Ct, and Rb(1.15)2Ct. Two homozygous combined translocation strains Rb(5.19)/(6.15) and Rb(5.19)/(6.15)/(8.17) are also maintained. All of these strains have now been demonstrated in our own and other laboratories to be capable of producing trisomy for the autosomes involved in the different translocations (see publications). Our previous studies of trisomy 19 demonstrated that affected fetuses were growth-retarded, and in certain crosses, had cleft palate. These findings have now been confirmed by Dr. Edward Bersu (Univ. Wisconsin, Madison) who now independently maintains strains capable of producing trisomy 19. He is currently working in our laboratory (IPA mobility assignment) to further delineate the trisomy 19 phenotype; he has already observed that the external phenotype of trisomic fetuses lags 24 hours behind that of normal littermates and that nucleated erythrocytes persist 48 hours beyond the usual time. Future studies include electron microscopy scanning of the trisomic embryo surface and observations of trisomic cells in vitro. Studies of trisomy 17 on a H-2^b background utilizing our Rb(8.17), H-2^b strain were planned for this year. However, these are postponed due to difficulty in maintaining that strain. At present, we are attempting to transfer the (8.17) translocation to the C57Bl/10 strain, which will require repeated backcrosses. If this is successful, future studies may include effect of trisomy 17 on H-2 and H-Y antigen expression and determination of in vitro characteristics of cells with trisomy 17.

Cytogenetic and Dermatoglyphic Studies of Patients.

These studies of selected patients, and their families whenever possible, emphasize the relationship between genetic disorders (including chromosomal) and abnormal growth and development. Of special interest are disorders affecting gonadal development and function, and correlation of phenotypic variation with cytogenetic and dermatoglyphic analyses. For these studies, a variety of techniques have been used for differential staining of chromosomes, including Giemsa-, quinacrine-, centromeric-, and BUdR-Hoechst 3325-banding. Results of several such studies have recently been published or are in press (see publications). Observations included determination of dermatoglyphic characteristics of pseudohypoparathyroidism and correlation with underlying osseous defects of the hand, further delineation of the phenotype and risk of gonadal tumor in individuals with 45,X/46,XY mosaicism and nonfluorescent Y chromosome, the first demonstration of consistent appearance of clones with autosomal aneuploidy in human fibroblast cultures (from a patient with 45,X/46,XY mosaicism, intestinal lymphangiectasia, and secondary immunodeficiency), and the first demonstration of mosaicism for H-Y antigen in gonadal tissue cultures from a 46,XX true hermaphrodite.

Our most recent patient studies followed the successful reproduction in our laboratory of the Yunis technique for prometaphase chromosome banding. This technique is superior to earlier techniques, since it produces more fully-extended chromosomes with delineation of greater numbers of bands. In collaboration with Dr. M. Kaiser (NEI), we have been able to use the Yunis method to confirm several previous reports that the syndrome of aniridia-Wilm's tumor-mental retardation is associated with an interstitial deletion of the short arm of chromosome 11. A report of these findings and their correlation in the patient and family with dermatoglyphic analyses and studies of the enzyme lactate dehydrogenase (LDH A and B subunits linked to chromosomes 11 and 12) is in preparation.

We are also conducting a study of aneuploidy and frequency of chromosomal aberrations in patients with Alzheimer disease, their families, and matched controls in collaboration with Drs. Goudsmit, Morrow, and Gajdusek (CNS, NINCDS). Patients are from two extensive kindreds in which this autosomal dominant disease is segregating; a few sporadic cases may also be studied. Chromosomes of affected members are also being analyzed with the Yunis technique, even though the lack of congenital anomalies in this disease (presenile dementia and premature death) makes a small chromosomal deletion or duplication unlikely. Viral etiology is also unlikely, because Dr. Gajdusek has so far been unable to transmit the familial disease in primates using tissue from affected members. Our main goals in this study will be to (1) Either confirm or deny a recent report from another laboratory claiming increased aneuploidy in Alzheimer disease, possibly due to microtubular pathology and disruption of the associated mitotic spindle, and (2) Make certain that there is no visible chromosomal lesion associated with the disease which might be of value for genetic counseling. All specimens will be collected by mid 1979 and analysis completed as soon as possible after that. Our results will be correlated with genetic markers, including erythrocyte antigens and HLA. Also, Dr. Goudsmit will be working in our laboratory to study chromosomes of affected family members with a new staining technique (energy transfer).

We are continuing to study fibroblasts of patients with proven intestinal lymphangiectasia with Dr. W. Strober (NCI). We are trying to determine if non-constitutional (secondary) clonal autosomal abnormalities are typical of cultures from these patients, as suggested in our initial report (see publications). The study now includes four patients, and hyperdiploid clones with trisomy 18 have been documented in cell lines from two of them; a third patient had no clones, and studies of the fourth are in progress. We suspect that these aneuploid cells may actually be present in vivo, since they were demonstrated after the first in vitro passage in the second patient. In vitro replicative lifespan of fibroblasts from all cases is also being determined, since preliminary observations of our first patient suggested it might be significantly decreased. In the last family, a history of disease in four successive generations was compatible with dominant inheritance, suggesting that some patients with sporadic intestinal lymphangiectasia may have fresh dominant mutations. This is a long-term project and we plan to study as many patients as possible.

Karyotypic Evaluation of Potentially Abnormal Tissues.

We have completed chromosomal studies of murine tumors in collaboration with Drs. D. Vembu and C. Boone (NCI). Together with their data, our results were interpreted as evidence of monoclonal origin of neoplastic tumors arising from the pre-neoplastic 10T 1/2 C3H parent cell line when implanted subcutaneously attached to plastic film (see publications). The results indicate that foreign body enhanced tumors may resemble some types of primary or spontaneous tumors, in that both may arise from a single abnormal cell.

Publications:

White, B. J., Crandall, C., Raveché, E. S. and Tjio, J.-H.: Laboratory mice carrying three pairs of Robertsonian translocations: establishment of a strain and analysis of meiotic segregation. Cytogenet. Cell Genet. 21: 113-138, 1978.

White, B. J. and Marx, S. J.: Dermatoglyphic and radiographic findings in a mother and daughter with pseudohypoparathyroidism. Clinical Genet. 13: 359-368, 1978.

Winters, S. J., Wachtel, S. S., White, B. J., Koo, G. C., Javadpour, N., Loriaux, D. L. and Sherins, R. J.: H-Y antigen mosaicism in the gonad of a 46,XX true hermaphrodite. New Engl. J. Med. 300: 745-749, 1979.

Boone, C. W., Vembu, D., White, B. J., Takeichi, N. and Paranjpe, M.: Karyotypic, antigenic, and kidney invasive properties of cell lines from fibrosarcomas arising in C3H/10T 1/2 cells implanted subcutaneously attached to plastic plates. Cancer Res. 39: 2172-2178, 1979.

White, B. J., Crandall, C., Flier, J. S., Raveché E. S. and Tjio, J. -H.: Cytogenetic studies of a male with sporadic intestinal lymphangiectasia: 45,X/46,XY mosaicism with pseudo- and hyperdiploid subpopulations in cultured tissues. Amer. J. Med. Genet. (in press).

Curtis, W. R. S., White, B. J., Lucky, A. W., Roche-Bender, N., Knab, D. R. and Johnsonbaugh, R. E.: Gonadal dysgenesis with mosaicism and a non-fluorescent Y chromosome: Report of two cases with correlation of clinical, pathologic, and cytogenetic findings. Amer. J. Obstet. and Gynecol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,010-03 LEP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Function of DNA virus genomes in animal cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Barrie J. Carter	Visiting Scientist	LEP	NIAMDD
Luis M. de la Maza	Visiting Scientist	LEP	NIAMDD
Catherine A. Laughlin	Staff Fellow	LEP	NIAMDD
Maureen W. Myers	Staff Fellow	LEP	NIAMDD
Francis T. Jay	Visiting Associate	LEP	NIAMDD
Carol J. Marcus	Staff Fellow	LEP	NIAMDD

COOPERATING UNITS (if any)
B. Moss, NIAID; W. Doerfler, Institute of Genetics-Cologne; D. Ward, Yale; T. Shenk and N. Jones, Connecticut; H. Westphal, NICHD.

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Chemical Pathology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	5.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are employing DNA viruses as molecular probes to study genome expression in human cells. We are studying intensively the structure and function of adeno-associated virus (AAV) since this virus has only a single gene. We are studying also adenovirus which is a more complex genome. Adenovirus is the helper virus for AAV multiplication. This helper relationship is being analysed. Also both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus this causes malignant transformation of the cell. AAV inhibits this transformation and also inhibits Ad12 oncogenesis in newborn animals. Thus AAV inhibits tumor induction. The mechanism of this inhibition of tumor induction is being studied at the molecular level in both cell culture and in animal experiments.

PHS-6040
Rev. 10-76)

Project Description:

Adeno-associated viruses (AAV) is a defective parvovirus. Because of its small DNA genome and unusual mode of segregation of complementary DNA strands into separate virions, AAV offers a unique situation amenable to biochemical analysis. AAV synthesizes only a few mRNA species (for its coat proteins). Thus, the AAV genome seems to consist of a single unique gene sequence enclosed by DNA regions containing reiterated sequences. We are studying AAV as a model system for eukaryotic gene expression. The main objectives of this study are: to analyse the structure of AAV DNA and obtain an accurate transcription map; to study the control of synthesis, post-transcriptional processing and modification and transport and degradation of AAV, to analyse replication of AAV DNA; and to study interactions between AAV and its helper adenovirus in both permissive and non-permissive cells. In order to analyse the interaction between AAV and adenovirus we require additional information directly relating to adenovirus function in infected cells. We have therefore initiated studies on various parameters of the adenovirus growth cycle.

Major findings since the last report are:

Adeno-associated virus interference:

Adeno-associated virus (AAV) grown in KB cells with a helper adenovirus, produced several classes of particles defined by their buoyant density in CsCl. The predominant density classes were referred to as AAV(1.45), AAV(1.41), AAV(1.35), and AAV(1.32), respectively, where the density of the particle is written in the parentheses. The AAV(1.45) and AAV(1.41) particles which contained standard genomes were the only infectious AAV particles. These infectious AAV particles exhibited autointerference. The light-density AAV(1.35) and (1.32) particles contained aberrant (deleted and/or snap-back) genomes. The light-density AAV particles were noninfectious but interfered with the replication of AAV(1.41). The interference was mediated intracellularly and resulted in inhibition of synthesis of standard (14.5 S) AAV genomes. In some cases, there was concomitantly an increased synthesis of aberrant, shorter AAV DNA. The inhibitory activity of the light-density particles was abolished by uv irradiation. These results show that the population of light AAV particles contained DI particles. The observed autointerference of AAV(1.45) or AAV(1.41) virus is postulated to be due to AAV DI particles. Replication of AAV DI genomes appeared to require the presence of replicating, standard AAV genomes. This is interpreted to mean that progeny strand replication of AAV requires an AAV-specified product, presumably the AAV capsid protein. In contrast to standard, infectious AAV, the AAV DI particles alone do not inhibit replication of the helper adenovirus. The structure of the AAV DI genome has important implications for packaging of viral DNA.

AAV DNA replication in vitro:

A complex which is active for *in vitro* synthesis of adeno-associated

virus (AAV) DNA was solubilized from Vero cells that were co-infected with AAV and either adenovirus (Ad5) or a herpes simplex virus type 1 (HSV-1) as the helper virus. The complexes from the Ad5 and HSV-1-infected cells sedimented at 23 S and 28 S, respectively. The optimal conditions for in vitro DNA synthesis for the two types of complex using the endogenous AAV template and the endogenous DNA polymerase, differed with respect to the effect of KCl and K_2SO_4 concentration. In addition the complex from HSV-1-infected cells, but not that from Ad5-infected cells, was inhibited by phosphonoacetic acid. Thus, the two complexes appear to contain different DNA polymerase activities. This was verified by phosphocellulose chromatography of the DNA polymerases solubilized from the isolated complexes. The major activity in the complex from HSV-1 infected cells was the HSV-induced DNA polymerase with lesser amounts of cellular DNA polymerase α and γ or both. The complex from the Ad5-infected cells contained mainly a cellular DNA polymerase γ.

This work and other studies suggest AAV2 and Ad5 DNA replication may share a common feature in a requirement for a DNA polymerase γ. Also, both Ad5 and AAV2 probably replicate via a single-strand displacement mechanism. Mitochondrial DNA also replicates by a single strand (D-loop) displacement mechanism and the mitochondria DNA polymerase is also a polymerase γ. This suggests the interesting speculation that DNA polymerase γ may be involved in strand displacement replication mechanisms.

AAV RNA Transcription: We have continued our studies on transcription of AAV mRNA in vivo using human KB cells infected with AAV and Ad2.

Previous studies, which showed that stable AAV RNA synthesized in vivo anneals only to the DNA minus strand between 0.18 and 0.88 map units, may not have detected unstable or nascent RNA. Therefore we developed a method of RNA preparation that would be expected to enrich for precursor RNA sequences. Whole cells were lysed with detergent and high salt and separated into supernatant and pellet (crude chromatin) fractions. These fractions were then separately deproteinized by proteolytic digestion and phenol extractions. DNA was removed from the preparation by two cycles of pancreatic DNase digestion and phenol extraction. Hybridization analyses of the RNA obtained from AAV/adenovirus-infected KB (human) cells revealed some AAV-specific RNA sequences that were not present in the mature 20S mRNA. These additional sequences were contained in AAV RNA molecules present in the pellet fraction, whereas the 20S AAV mRNA accumulated in the supernatant. A species of AAV-specific RNA (about 22S), which was associated only with the pellet fraction and was labeled only after a short pulse, appeared to have a kinetic relationship with the more stable cytoplasmic 20S mRNA. These putative AAV mRNA "precursors" and precursor sequences were not observed previously when conventional methods were used to obtain RNA from either whole cells or isolated nuclei.

We have now mapped the cytoplasmic species of AAV RNA by analysing RNA-DNA heterduplexes either by electron microscopy or by the nuclear digestion procedure of Berk and Sharp. This work revealed 4 species of AAV mRNA containing the same spliced out region but differing in the length of the 5' leader sequence.

These studies establish the parvoviruses as an excellent system, because of their simplicity, with which to attempt to obtain correct initiation and/or post-transcriptional processing of RNA in in vitro systems.

Publications:

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F. T. Jay, C. A. Laughlin, W. J. Cook, L. M. de la Maza and B. J. Carter: Adeno-associated virus RNA synthesis in vivo and in vitro. In Replication of Mammalian Parvoviruses (eds. D. Ward and P. Tattersall) Cold Spring Harbor Laboratory, New York, p 385-398, 1978.

L. M. de la Maza and B. J. Carter: DNA structure of incomplete AAV particles. In Replication of Mammalian Parvoviruses (eds D. Ward and P. Tattersall) Cold Spring Harbor Laboratory, New York, p 193-204, 1978.

R. Armentraut, R. Bates, K. Berns, B. Carter, M. Chow, D. Dressler, K. Fife, W. Hauswirth, G. Hayward, G. Lavalle, S. Straus, S. Rhode, P. Tattersall and D. Ward: A standardized nomenclature for restriction endonuclease fragments. In Replication of Mammalian Parvoviruses (eds. D. Ward and P. Tattersall) Cold Spring Harbor Laboratory, New York, p 523-526, 1978.

B. J. Carter, C. A. Laughlin, L. M. de la Maza and M. W. Myers: Adeno-associated virus autointerference. Virology 92: 449-462, 1979.

C. A. Laughlin, M. W. Myers, D. L. Risin and B. J. Carter: Defective-interfering particles of the human parvovirus adeno-associated virus. Virology 94: 162-174, 1979.

F. T. Jay, L. M. de la Maza and B. J. Carter: Parovirus RNA transcripts containing sequences not present in mature RNA. A method for isolation of putative mRNA precursor sequences. Proc. Nat. Acad. Sci. USA. 76: 625-629, 1979.

H. Handa and B. J. Carter: Adeno-associated virus DNA replication complexes in Herpes Simplex virus or adenovirus infected cells. J. Biol. Chem. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 21,012-02 LEP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cytogenetics and Immunogenetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. S. Raveche	Research Chemist	LEP	NIAMDD
OTHER:	J. H. Tjio	Chief, Section on Cytogenetics	LEP	NIAMDD

COOPERATING UNITS (if any)
A. Steinberg, NIAMDD
O. Alabaster, NCI
R. Viergersky, Walter Reed, Wash., D. C.
D. Shreffler, Washington University School of Medicine, St. Louis, Mo.

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Cytogenetics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Application of differential staining techniques for chromosome analysis.

2. Studies of the inheritance of several autoimmune immunological traits including antibodies cytotoxic for T lymphocytes and reactive with single stranded DNA.

3. Studies of inheritance of hyperdiploidy.

4. Analysis of sex hormone modulation of immune responses.

5. Use of flow cytometry in the analysis of aneuploid cells.

PHS-6040
(Rev. 10-76)

Project description:

Murine Immunogenetic Studies:

The appearance of naturally occurring thymocytotoxic autoantibodies (NTA) and spontaneously produced antibodies to single-stranded DNA (ssDNA) are characteristic of the autoimmune disease of NZB mice. These autoantibodies were found to be inherited traits but influenced by sex hormones. In F_1 hybrids of the NZB strain and a normal strain, we found that testosterone suppresses the formation of NTA. However, in the parent NZB strain no sex difference or suppression by testosterone of NTA production was observed. From various crosses we are postulating on androgen insensitivity in NZB mice. In support of such a concept, in preliminary work with Dr. Donald Shreffler, Washington Univ., St. Louis, we have studied a serum protein (Slp) which is also testosterone sensitive. Most strains show a sex difference with regard to slp production but NZB animals do not. We are currently studying this in greater detail to determine if androgen insensitivity is related to autoimmune disease.

Experiments are currently being performed to investigate which cells have antigens reactive with NTA. Initial studies have shown that NTA is highly cytotoxic for immature lymphocytes found in the thymus. To confirm and extend those studies, the lectin, peanut agglutinin has been employed. This lectin agglutinates immature cells. We have found that cells agglutinated by peanut lectin are readily killed by NTA whereas cells not agglutinated are less readily killed. This is in agreement with our previous finding that Con A stimulated thymocytes are also resistant to lysis by NTA.

Murine Cytogenetic Studies:

Experiments in collaboration with Dr. A. Steinberg and Dr. J.H. Tjio (NIAMDD) to determine the effects of genetic background on the expression of hyperdiploidy in various crosses with NZB mice are currently underway. We have completed analysis of crosses of the NZB strain with the normal DBA/2 strain and found that hyperdiploidy was inherited as a recessive trait and not related to the presence of other immunological abnormalities including splenomegally, hypergammaglobulinemia, spontaneous antibodies cytotoxic for T cells and reactive with single-stranded DNA. We are currently studying crosses of NZB with various other strains to determine the mode of inheritance of these abnormal traits.

In addition, we are currently studying the ability of hyperdiploid cells to repopulate recipient spleens. Hyperdiploid spleens are identified by analysis of partial spleen preparations, if present the remainder of the spleen is transferred to various recipients. The excessive proliferation of NZB spleen cells can be studied in this manner.

Flow Cytometric Studies:

Experiments are currently being performed with Dr. O. Alabaster (NCI)

to investigate the cell type of aneuploid NZB spleen cells. This is being performed by flow cytometric techniques which use fluorescent dye systems to measure the DNA content per cell. By this technique, a fluorescence distribution for a particular population of cells can be obtained. In a population of NZB cells containing hyperdiploid cells there are two G_1 peaks. One corresponding to the normal cells and the second G_1 peak of greater fluorescence corresponding to the aneuploid G_1 cells. With such analysis we determined that the aneuploid cell present in NZB spleens is a "null" cell, neither a T cell nor a B cell. We are currently studying cell cycle kinetics by this technique.

Hormone Receptors:

In collaboration with Dr. R. Viergersky, Walter Reed Army Medical Center, we are studying sex hormone receptors in the thymus. Since sex hormones profoundly effect the immune response, we are searching for the mechanism by which hormones mediate this action. In preliminary work, we have identified a ß-estradiol specific cytosol receptor in the murine thymus. We are investigating the size, binding affinity and specificity of this receptor.

Thymus Transplants:

Because the thymus seems to have sex hormone receptors, we are initiating experiments involving neonatal thymectomies followed by opposite sex thymus grafts. By this technique, we hope to further investigate the postulated immune androgen insensitivity of NZB mice and the role of the thymus in sex differences.

Hormones and X-linked Immune Response Genes:

These studies were performed to test the previously advanced hypothesis that the antibody response to ssDNA is controlled by an X-linked immune response response gene. THe antibody response to single-stranded DNA (ssDNA) complexed to MBSA was studied in NZB, DBA/2, F_1 reciprocal hybrids, and backcross mice. We found that the anti-ssDNA response was not linked to genes located on the X chromosome, but rather that anti-ssDNA production was regulated by sex hormones. Reciprocal F_1 hybrid males with either an NZB or a DBA/2 X chromosome were low responders; however, either castration or castration plus estrogen implants significantly increased the amount of antibody produced. Testosterone implants were immunosuppressive in both females and males. Backcross studies suggested that the ability to respond to ssDNA is inherited as an autosomal dominant trait that is further regulated by sex hormones.

Control studies were performed in hybrids of the CBA/N strain, which has a well established X-linked defect in the ability to respond to a variety of thymic independent antigens including polyriboinosinic·polyribocytidylic acid (rI·rC). F_1 hybrid males that received the CBA/N X chromosome were low-

responders to rI·rC but testosterone implants in females decreased the amount of antibody produced.

These studies indicate that it is possible to separate sex hormone effects from X-linked genetic effects on immune responses. We found that the response to ssDNA is controlled by sex hormones but not an X-linked immune response gene.

Publications:

Steinberg, A. D., Klassen, L. W., Raveche, E. S., Gerber, N. L., Reinertsen, J. L., Krakauer, R. S., Ranny, D. F., Gershwin, M. E. and Reeves, J. P.: Study of multiple factors in the pathogenesis of autoimmunity in New Zealand mice. Arthritis and Rheum. 21: 190-201, 1978.

White, B. J., Crandall, C., Raveche, E. S. and Tjio, J. H.: Laboratory mice carrying three pairs of Robertsonian translocations; establishment of a strain and analyses of meiotic segregation. Cytogenetics and Cell Genetics. 21: 113-138, 1978.

Raveche, E. S., Tjio, J. H. and Steinberg, A.D.: Genetic studies in NZB mice II. Hyperdiploidy in the spleen of NZB mice and their hybrids. Cytogenetics and Cell Genetics. 23: 182-193, 1979.

Raveche, E. S., Tjio, J. H., Steinberg, A. D.: Genetic studies in NZB mice III. Induced anti-nucleic acid antibody production. J. of Immunology. 122: 1454-1460, 1979.

Raveche, E. S., Tjio, J. H., Steinberg, A. D.: Studies of the effects of sex hormones on autosomal and X-linked genetic control of induced and spontaneous antibody production. Arthritis and Rheumatism (in press).

Raveche, E. S. and Steinberg, A. D.: Lymphocytes and lymphocyte function in SLE. In Clinics in Hematology ed. H. Fudenberg (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,013-02 LE

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Electron Microscope Investigations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Howard A. Bladen	Research Biologist	LEP	NIAMDD
OTHER:	Jacqueline Henson	Histopathologist	LEP	NIAMDD

COOPERATING UNITS (if any) L. M. de la Maza, LEP, NIAMDD; T. Chai, LBM, NIAMDD; C. Tabor, LBP, NIAMDD; R. Maheshwari, LEP, NIAMDD; C. Creveling, LC, NIAMDD; E. Grollman, LPP, NIAMDD; B. Rosset, LEB, NIAMDD.

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

During the last year this unit has been involved in studies with several investigators within NIAMDD. Some of these have been on a continuing basis while others have been short term and involved essentially screening of samples.

PHS-6040
(Rev. 10-76)

Project description:

Dr. Chai requested that we investigate the morphological characteristics of some psychrophilic Moraxella cells of marine origin to determine the cause of the development of pleomorphic shapes after storage at 2°C. Results demonstrated that degradation of the peptidoglycan layer of the cell envelope is probably one of the causes resulting in cell lysis and changes in cell shape.

Various strains of E. coli were negatively stained and examined by electron microscopy to determine the presence or absence of what appeared to be an incomplete bacteriophage. This project is on a continuing basis with Dr. C. Tabor.

A project is in process with Dr. de la Maza to characterize the DNA from incomplete adeno-associated virus (AAV) particles that arise during growth in the presence of helper adenovirus. Physical measurements of the DNA from numerous samples after application of a spreading technique and rotory shadowing has allowed more exact characterization of the viral DNA with regard to size and structure.

Previous results of Dr. Maheshwari showed that as much as 200 fold inhibitions of infectious VSV was achieved in cultures of Ly cells treated with 30 reference units/ml of interferon as judged by infectivity assay; however, virus particle production, as judged by chemical methods was inhibited a maximum of 10 fold. These results suggest that IF treatment produces particles with low infectivity. We are currently investigating by electron microscopy the number and type of particles in both IF treated and control virus preparations. Preliminary results suggest that there is a 10-25 fold reduction in particles in preparation of virus from IF treated cells.

Collaborated with C. R. Creveling (LBC) on an investigation of the morphological characteristics of post-synaptic vesicular structures from rodent cerebral cortex. Amine-synaptic structures present in homogenates and to permit a partial isolation of these entities. EM studies were performed at various stages of purification in order to identify which of the structures present in the cruel homogenate contain the cyclic AMP generating system.

Publication:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 21,014-02 LEP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Control of viral genetic function in interferon-treated and virus-infected animal cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Radha Maheshwari	Visiting Fellow	LEP	NIAMDD
OTHER:	Robert M. Friedman	Chief, LEP	LEP	NIAMDD
	David Epstein	Staff Fellow	LEP	NIAMDD
	Chris Czarniecki	Staff Fellow	LEP	NIAMDD

COOPERATING UNITS (if any)

S. Arya, NCI Paul Torrence, NIAMDD Margaret Johnston, NIAMDD
A. K. Bandyopadhyay, NCI; A. Dempsey, NCI.

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Chemical Pathology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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3-1/3	2-1/3	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Interferon treatment of cells producing murine luekemia viruses (MLV) or mouse mammary tumor virus (MMTV) resulted in marked decrease in virus yields. In some system this appeared to be due to an inhibition in virus budding from the plasma membrane of the cell; in others, virus with marked decrease in infectivity was produced. Several proteins of MLV seem to be altered in virus derived from interferon-treated cells. In addition, in an RNA virus from an entirely different group, vesicular stomatitis virus (VSV), particles of low infectivity are produced after interferon treatment. In this system, there appears to be an inhibition in the incorporation of the viral glycoprotein into the virion. These changes may be related to interferon induced alterations in the cell plasma membrane.

In addition, the mechanism of interferon-induced inhibition of virus protein synthesis in cell-free systems is being studied.

PHS-6040
(Rev. 10-76)

Project description:

Replication of endogenous type C RNA tumor viruses was sensitive to interferon treatment. Interferon treatment also caused a 10 to 20-fold increase in intracisternal A-particles in erythroleukemic cells infected with Friend virus; the A-particles may have been immature C-particles in these systems. Mouse mammary tumor virus (MMTV) (B-particles) production was also inhibited by interferon treatment.

Inhibition of RNA tumor virus production was not correlated with inhibition of at least some intracellular steps in virus replication. In interferon-treated AKR cells in which there was marked inhibition of production of both endogenous MLV particles and infectious MLV, the intracellular concentration of viral p30, group-specific (gs), antigen was unaffected or even increased. SEM and transmission micrographs clearly indicated that in mouse systems the number of surface-associated MLV particles was increased. Similar findings have been made in MMTV virus producing cells (A. Dempsey, personal communication). This may have been the reason why, in the case of MLV, cell-associated p30 (gs) antigen was increased in these systems after interferon treatment. Interferon induced inhibition of MLV production probably does not involve inhibition of virus protein synthesis.

Viral RNA synthesis has also been studied. In interferon-treated mouse cells infected with Moloney MLV or AKR-MLV, the concentration of virus-specific RNA was approximately the same as in untreated cells. The rates of viral RNA synthesis were also about equal. Substantially, the same findings have been made in chronic MMTV infection after interferon treatment (S. Arya, personal communication).

In both chronic and acute Moloney MLV infection in TB cells there was a marked inhibition in the infectivity of the MLV particles produced. It would seem, therefore, that although in some systems interferon treatment resulted in marked inhibition of virus release, in others, particle production was almost normal, but the virus released was quite deficient in infectivity.

One possible basis for the low infectivity was the presence in moderate concentration of a glycoprotein of about 90,000 in molecular weight (gp 90) in Moloney MLV released by interferon-treated TB cells. This protein was only a trace component in MLV produced in control cells; antiserum to the major glycoprotein, gp 70, of MLV cross-reacted with the gp 90 in MLV produced by interferon-treated TB cells (E. Chang, personal communication).

The glycoprotein may not be the only site of interferon action in non-infectious MLV particles released after interferon treatment. There also appears to be a defective MLV protein-protein complex between the viral p 30 antigen and reverse-transcriptase in Moloney MLV produced in interferon-treated TB cells. This defect may contribute to the decreased

infectivity of such virus particles.

One important question about this work is whether such findings are limited to murine RNA tumor virus systems, or whether they are operative in the case of viruses from other groups. We are, therefore, in the process of studying vesicular stomatitis virus (VSV) produced by interferon-treated L-cells (Ly strain). We have so far obtained the following results: in Ly cells treated with 30 units/ml of interferon there was an approximately 200-fold reduction in the titer of infectious VSV production; however, virus particle production, as measured by VSV particle-associated RNA, N or M protein, or transcriptase was inhibited by a maximum of 10-fold by this concentration of interferon. A greater reduction (25-fold) was however noted in the glycoprotein content of VSV from interferon-treated cells. These results suggested that IF-treated cells produce VSV particles with low infectivity (R. Maheshwari + R.M. Friedman, J. Gen. Virol., in press).

Only 42S viral RNA was detected in the VSV produced by interferon-treated cells; these particles did not interfere with the growth of wild-type VSV in BHK or Ly cells, nor did they induce interferon in culture. The findings suggested that these particles are not defective interfering forms of VSV. The results in many respects resemble those previously reported in IF-treated cells infected with murine leukemia viruses. We concluded, therefore, that such findings are not limited to murine RNA tumor virus systems; the inhibition of some membrane-associated viruses by treatment with relatively moderate concentrations of interferon may be a wide-spread phenomenon which is closely related to functional abnormalities in the proteins incorporated into non-infectious virions produced by such cells. Since many studies have demonstrated that VSV particles with reduced amounts of glycoprotein are low in infectivity, it is likely that at least some of the reduced infectivity of VSV particles produced by interferon-treated cells was due to the reduced amount of this protein incorporated into such particles. It is possible induced changes which have been reported to occur in the plasma membrane of interferon-treated cells may account for the alterations in infectivity of both VSV and murine RNA tumor viruses, since these viruses bud from the cell surface as a terminal step in the replication process.

Publications:

Friedman, R. M.: The mechanism of action of interferon: possible primary effects on membranes. Horizons in Biochem. and Biophys. 5: 281-313, 1978.

Friedman, R. M.: Interferon and cancer treatment. Ca - a Cancer Journal for Clinicians. 28: 278-283, 1978.

Torrence, P. F. and Friedman, R. M.: Are double-stranded RNA-directed inhibition of protein synthesis in interferon-treated cells and interferon

induction related phenomena? J. Biol. Chem. 254: 1259-1267, 1979.

Bandyopadhyay, A. K., Chang, E. H., Levy, C. C. and Friedman, R. M.: Structural abnormalities in murine leukemia viruses produced by interferon-treated cells. Biochem. Biophysical Res. Comm. 87: 983-988, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,015-02 LEP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on Rouse sarcoma virus and Simian virus 40

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Francis T. Jay	Visiting Associate	LEP	NIAMDD
Luis M. de la Maza	Visiting Scientist	LEP	NIAMDD
Barrie J. Carter	Visiting Scientist	LEP	NIAMDD
Robert M. Friedman	Chief, Laboratory of Experimental Pathology	LEP	NIAMDD

COOPERATING UNITS (if any)
G. Jay, NCI
A. Levine, NCI
C. Chang, NCI

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Chemical Pathology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In studies on SV40 early antigens, Ad2^{+}ND$_{2}$ induced in infected cells two SV40 specific polypeptides of 42,000 and 56,000 molecular weight; Ad2^{+}ND$_{1}$ induced a 28,000 molecular weight peptide. The Ad2^{+}ND$_{1}$ segment is found to be sufficient to induce the SV40-specific tumor antigen in mice.

With 2-dimensional gel electrophoresis a 4th AAV structural protein (VP) was detected. Each of the four VP's (m.w. 86.5, 72.5, 61.5, 60.5) contain multiple iso-electric species. While VP 1, 2 & 3 share many tryptic peptides, at lease, VP 1 & 3 each contain unique tryptic peptides. Virion proteins as well as cell free translated proteins are presently being analysed to test whether the VP's are overlapping or that frame shift also plays a part as a method apparently to conserve DNA by this virus.

PHS-6040
(Rev. 10-76)

Studies on Simian Virus 40 early antigens.

We have previously reported that $Ad2^{+}ND_2$ induced in infected cells two SV40 specific polypeptides of 42K and 56K mw; and that $Ad2^{+}ND_1$ induced a 28K polypeptide. Their biosynthesis, subcellular localization were studied and discussed with respect to their possible functions. We have performed a systematic study to correlate the immunologically-detected SV40 early antigens with the biochemically detected polypeptides induced in the $Ad2^{+}ND$ hybrids and to study the biological activities of these antigens and their interactions with the host in order to understand the biological controls which affect the outcome of a viral infection. Cells transformed by simian virus 40 (SV40) possess a tumor-specific transplantation antigen(s) (TSTA) which has the property of immunizing animals against syngeneic tumor challenge. We find that the early SV40 DNA segment present in $Ad2^{+}ND_1$, is sufficient to induce this SV40-specific TSTA in BALB/c mice. Moreover, studies on the intracellular distribution of TSTA activity in $Ad2^{+}ND_1$-infected cells, as determined by the ability of various subcellular fractions to immunize mice against syngeneic tumor challenge, have suggested a correlation between this biological activity and the presence of the SV40-specific 28,000 M_r protein coded by this hybrid virus. Both the TSTA activity and the 28,000 M_r protein are found in the plasma membrane fraction and in the perinuclear region of infected cells, but are virtually undetectable in the cytoplasmic fraction. Using a hamster antitumor serum that can specifically immunoprecipitate the 28,000 M_r protein, we are able to demonstrate a loss of TSTA activity concomitant with the removal of this SV40-coded protein. Thus, it appears that the antigenic determinants responsible for SV40-specific tumor rejection in mice are contained within the 28,000 M_r polypeptide coded by the early SV40 segment which extends from 0.17 to 0.28 map units.

Adeno-Associated Virus Proteins

It has been reported that Adeno-Associated Virus (AAV) has three structural proteins associated with the virion. However, the combined molecular weights is larger than the coding capacity of the AAV genome. Our objective is to find out how these structural proteins are coded by the virus by studying their relationships to see if they have overlapping peptide sequences or that the virus uses different reading frames to make several proteins from minimal genome length. Our initial results indicated that first, AAV has 4 structural proteins instead of three as previously reported, distinguishable by their molecular weight designated as VP1, VP2, VP3, and VP4 with molecular weights of 86.5, 72.0, 61.5 and 60.5. Secondly, while VP1, 2 and 3 share many tryptic peptides, at least VP1 and 3 each has unique peptides. Finally, 2D gel analyses of these viral proteins showed that all of these VPs exhibit multiple iso-electric species. We are presently trying to study individual VP species by peptide mapping to see whether they are completely overlapping or not or that frame shift plays a part in conserving genome size. To tackle this problem, we are synthesizing these VPs in cell free system for 2D gel and peptide map analyses.

Publications:

Jay, G., Jay, F. T., Chang, C., Levine, A.S., and Friedman, R. M.: Induction of SV40-specific tumor rejection by the $Ad2^{+}ND_{2}$ hybrid virus. J. Gen. Virol. (in press).

Jay, F. T., Laughlin, C.A., Cook, W. J., de la Maza, L. M. and Carter, B.J.: Adeno-associated virus RNA synthesis in vivo and in vitro. In Parvoviruses: Replication and Biological Properties. (ed. Ward, D. C. & Tattersall, P.) Cold Spring Harbor Laboratory, 1978.

Jay, F. T., de la Maza, L.M. and Carter, B.J.: Parvovirus RNA transcripts containing sequences not present in mature mRNA: A method for the isolation of putative mRNA precursor sequences. Proc. Natl. Acad. Sci. USA 76: 625-629, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 21,016-01 LEP

PERIOD COVERED
January 1, 1979 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Purification of Messenger RNA for Human Interferon.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Robert M. Friedman — Chief, Laboratory of Experimental Pathology — LEP — NIAMDD

COOPERATING UNITS (if any)

Michael Hitchcock, NIAMDD
Shelby Berger, NCI
Kathryn Zoon, NIAMDD
Esther Chang, NCI

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Section on Chemical Pathology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The messenger RNA is obtained from human Namalva (lymphoblastoid) cells. Partial purification of this mRNA has led to about a 90-fold enrichment in our best preparations. The enriched preparations are transcribed by an RNA tumor virus reverse transcriptase and double stranded DNA is made from this.

PHS-6040
(Rev. 10-76)

LABORATORY OF CHEMICAL BIOLOGY, NIAMDD
ANNUAL REPORT: OCTOBER 1, 1978 - SEPTEMBER 30, 1979

The Laboratory of Chemical Biology has, during fiscal 1979, continued its emphasis on the conformation of macromolecules and their interaction with both other proteins and small ligands. The physical bases of the folding and stabilization of protein molecules is being studied in complexing systems of fragments prepared from parent native molecules and in several cases, as well, with semisynthetic complexes in which one of the components has been synthesized in a variety of analog forms. These experiments begin to give direct information about how certain organized, internally cooperative portions of proteins can function as nucleating sites for folding and centers for stabilization in the completed molecules. Most of these studies are concerned with the process, rather than the biology of the macromolecules in question. However, in certain other experiments, direct questions of biological interest can be asked and such more directed efforts are being made on normal and abnormal forms of hemoglobin, the neurophysin-oxytocin or -vasopressin system and with human interferon.

An ongoing investigation, with very direct biological implications, uses as the test object, Paramecium multimicronucleatum. This organism is being studied at the protein chemical level in connection with characterization of the surface antigens and of the mating-type substances responsible for cell-cell interaction in these protozoa.

Running through many of the research efforts being carried out in LCB is the theme of affinity chromatography, both of the immunochemical and ligand-specific varieties. This technique has found broad application in the isolation of proteins and parts of proteins as well as in the estimation of the affinities between molecules.

I. Chemistry, function and three-dimensional conformation of macromolecules

A. The dynamics of protein conformation (in continuation of studies described in earlier reports)

(1) As a ground work for the study of the relationship between the amino acid sequence and the stabilizing energy of the three-dimensional structure, the X-ray diffraction of crystals of Nuclease-T bound with ligands has been measured at 2 to 4°C to resolution between 2 and 3A using a precession camera in order to determine the atomic coordinates. (2) On the basis of measurements using a stopped flow technique, folding of the nuclease A portion of nuclease B appears to be thermodynamically and kinetically independent of the 19 residues at the NH_2-terminus. (Staphylococcal nuclease B is a synthetic precursor of the normally isolated nuclease A and differs from nuclease A by the addition of 19 amino acid residues at the amino terminus. This additional stretch of chain is, for the most part, removed before secretion of nuclease A into the culture medium.) (3) The equilibrium constant of Nuclease-(1-126) with deoxythymidine-3',5'-diphosphate in the presence of 0.01 M Ca^{2+} has been measured by a dialysis equilibrium method. Nuclease-(1-126) was shown earlier to have a very low level of enzyme activity in spite of lacking 23 residues at the C terminal end. The

truncated derivative is almost completely unfolded in solution and the present data reaffirm that the molecule must be able to assume a "native format" to a slight extent in the presence of substrate and strong inhibitors. (Taniuchi, Parker, Davis)

Knowing that the two-fragment complex, Nuclease-T, crystalizes in a form that is isomorphous with unfragmented native nuclease permits the design of experiments to prepare semisynthetic nuclease-T in which the smaller parts of the complex may be made with deliberate variations in sequence with the purpose of elucidating the roles of specific residues in function and stabilization of internal structure. It is of interest, in connection with the permissibility of changes in covalent structure in relation to kinetic parameters of folding, that nuclease B, with its 19 additional residues, folds in a two-stage time course with rates essentially identical to those observed with nuclease A.

An even more demanding set of studies has involved active three-fragment complexes, one reported in the annual report for fiscal '76 (derived from the nuclease molecule) and the second, first observed at the Rockefeller Institute, derived from pancreatic ribonuclease. Once again, one of the three fragments in both systems has been made synthetically and recombined with the other two native fragments. The above experiments have strong bearing on the nature of the interatomic interaction that operate in formation of unique three-dimensional structure. Kinetic and thermodynamic details are being further examined using X-ray crystallography, measurement of fragment exchange, and estimating activation energies during the folding and unfolding of fragment complexes. (Juillerat and Taniuchi)

B. Folding of two-fragment complexes of cytochrome c

Biologically functional, non-covalently linked complexes of cytochrome c are generated from several combinations of overlapping and non-overlapping fragments. The use of fast kinetic techniques to study the dynamic events leading to the formation of an ordered structure suggested that the process occurs in at least two distinct phases. An initial, second order reaction was observed in which the fragments combined to form a product having a structure reasonably similar to native cytochrome c. Additional evidence indicated that achievement of a native-like ligation state of the heme iron involves a distinct kinetic event occurring after the second order reaction. This proposition has been tested by using chemically modified derivatives of the original fragments. These modifications prevent formation of a ligation state identical to that of native cytochrome c. In one instance the iron atom is removed from the heme moiety and in the other the methionine residues are converted to methionine sulfoxide. These derivatives exhibit second order kinetic behavior nearly identical to that of the original fragments. This provides strong support for the idea that formation of the native-like structure occurs in at least two kinetically distinct phases. (Parr and Taniuchi)

C. Cytochrome c biosynthesis

In order to find a hypothetical enzyme (cytochrome c synthetase) which

would catalyze the covalent bonding of heme to apocytochrome c, Saccharomyces cerevisiae has been chosen as an enzyme source. Synthesis tests have been carried out using ^{125}I labeled apocytochrome c and heme as substrates, and the yeast mitochondria as a source of enzyme enriched cellular fractions. The extent of labelled apoprotein incorporation into the fraction of cytochrome c added as the carrier has been as much as 7% (0.35 nmol) of the extracted radioactive material. This radioactive material, resembling cytochrome c, will be further investigated in order to establish the exact nature of the observed phenomenom. (Basile and Taniuchi)

D. Folding in three-fragment complexes of cytochrome c

ytochrome c, a biologically active, three-fragment complex composed of heme fragment (1-25)H, and apofragments (28-38) and (56-104) was developed. This complex was obtained by a limited tryptic digestion of the parent complex formed by heme fragment (1-25)H and apofragments (1-65) and (39-104). This three fragment complex of cytochrome c is expected to serve as an excellent test object to investigate whether a global potential function is operative in the ordered structure. Measurements of the fragment exchange rate would give an unequivocal answer to the question as to whether the binding energy of each fragment is derived from the same global cooperative interaction. Since one of the three fragments, (28-38) can be readily synthesized, the system can also be used for the study of the effect of substitution of residues on dynamics and structure-function relationships of cytochrome c and would help in understanding the reason why certain specific residues are evolutionarily invariant. (Juillerat and Taniuchi)

E. A study of peptide-protein interactions using a model semisynthetic RNase-S'

Based on experimental and theoretical studies reported to date on RNase-S', it was postulated that the minimum structural information expressed in the N-terminal sequence of RNase-S is an α-helix on which the residues Phe and Met are specifically positioned for maximal apolar interactions with RNase (21-124). To test this postulate, a model dodecapeptide was previously designed and synthesized by solid phase method. Properties of this peptide in terms of its solution conformation and its affinity toward S-protein were characterized. Further, the ability of this peptide to help direct the regeneration of active RNase (21-124) tertiary structure, after the initial scrambling of the disulfide bonds, was determined. For crystallographic structural analysis, purified model sRNase-S' was successfully crystallized in the same space group as native complex. Efforts were made to crystallized synthetically derived native and model analogue peptides. At present, crystals of [Ala 13] sRNase (1-15) have been obtained. For a continuing study on RNase enzymatic mechanism, the characterization of the residual activity found for [4-F-His12, des 16-20]sRNase-S' and covalent [4-F-His^{12}]sRNase-A is in progress. (Chaiken, Komoriya)

F. Antibodies against neurophysin and other proteins and their use for conformational and biosynthetic studies

This project is part of a general program to use specific antibodies as tools to study the chemical basis and biosynthetic origins of native protein conformation.

Purified neurophysin antibodies have been used to identify biosynthetic precursors of neurophysins (and the closely associated peptide hormones oxytocin and vasopressin) which are produced in cell-free synthesis in the presence of bovine hypothalamic mRNA. The nature of recognition of neurophysins and precursors with the purified antibodies is being studied.

Antibodies prepared against thermolysin and two large fragments of this protein have been used in immunodiffusion assays and radioimmunoassays to study conformational properties of the native protein as well as the fragments.

Affinity chromatographic techniques have been used to isolate ribonuclease antibodies specific for the S-peptide region (residues 1-20). Competition radioimmunoassay experiments are being carried out to determine the precise specificity of the antibodies and to try to use these to design semisynthetic proteins with altered antigenicity. (Chaiken, Seeman, Sobieski)

G. 1. Characterization of bovine neurophysin - neurohypophyseal hormone complexes

As part of a continuing study of the neurophysin-peptide hormone system, the tripeptide analogue of vasopressin, L-methionyl-L-tyrosyl-L-phenylalaninamide, has been modified for use in photoaffinity labeling studies of the hormone binding site on neurophysin. The photo-sensitive peptide, L-methionyl-L-tyrosyl-p-azido-L-phenylalaninamide, was found by equilibrium dialysis in the absence of light to be reversibly bound to bovine neurophysin II. The dissociation constant, stoichiometry, pH-dependence and competition properties with hormone analogues indicate that the interaction is similar to the interaction of hormone with neurophysin.

Covalent complexes resulting from irradiation were fractionated on an affinity matrix. Investigation of the specifically labeled complex which would not bind to the affinity matrix revealed a 1:1 stoichiometry of incorporation and when oxidized, digested with trypsin and subjected to peptide mapping, affinity label remained attached to the peptide mode up of residues 44-66. Details of the site of attachment within this peptide will be studied by sequencing techniques. Irradiation of photoaffinity label with neurophysins from other species produces similar complexes and demonstrates the general applicability of this system. (McCormick, Chaiken)

G. 2. Quantitative affinity chromatographic studies of interacting macromolecular systems

This project is part of a continuing program to develop and use bioaffinity chromatography to determine quantitative characteristics of macromolecule-involved interactions. A major study was completed on the quantitative expression in affinity chromatography of antibody polyvalency in antigen binding. Studies also were completed on the interaction of psychoactive drugs with the multisubunit enzyme, bovine glutamate dehydrogenase, and on the interaction of peptide ligands with bovine neurophysins. (Chaiken, McCormick)

II. Enzymatic resynthesis of peptide bonds in organic media

In order to extend capabilities in the study of the effect of amino acid sequence on protein function, a novel enzymatic resynthesis method was applied to several noncovalent fragment complexes. Substitution of water by partially nonaqueous solvents (e.g 90% glycerol) in systems of limited proteolyses, thermodynamically stabilizes the peptide bond and allows resynthesis (30-50%). A noncovalent complex of ribonuclease S-protein and 4-Fl-His^{12}-S-peptide was converted to a covalent form, allowing the study of catalysis at a low pH where the complex would have dissociated. A counterpart system where a peptide, 4-Fl-His^{119} (116-124) was chemically synthesized and complexed to ribonuclease-(1-115) provided additional information to suggest that both His^{12} and His^{119} are general catalytic acid bases. A two-fragment staphylococcal nuclease complex was "restitched" with the exclusion of loop residue, Lys^{49}. This completely abolished activity, suggesting the importance of the integrity of the loop for the correct conformation at the active site. A cytochrome c complex and a prothrombin 1 complex were also restitched, demonstrating the potential for extended studies of covalent hybrids of these proteins. (Homandberg, Chaiken)

III. Studies on normal, fetal and sickle hemoglobin

A. Hemoglobin oxygen affinity: immunochemical studies

Using non-precipitating monospecific antibodies we have been studying the conformation of hemoglobins in solution. Hemoglobin A_0 and A_2 have been isolated, purified and isotopically labeled either by carbamoylation with K^{14}CNO or by reductive alkylation with tritiated borohydride. A specific subpopulation of anti-hemoglobin antibodies has been isolated by affinity chromatography using a synthetic peptide corresponding to α(129-141) and has been used in a radioimmunoassay to study conformational changes that occur at the carboxy-terminus of α chains during oxygenation. These antibodies which bind with an affinity of 5×10^8 M^{-1} to CO-liganded ^{14}[C] hemoglobin increase the oxygen affinity of hemoglobin and, thus, act as a linked function. A second subpopulation of antibodies has been isolated that react specifically with 3[H]-hemoglobin A_2. These antibodies will be used in a radioimmunoassay to detect hemoglobin A_2 levels in serum and cultured erythropoietic cells as well as to differentiate A_2 from other normal and mutant hemoglobins. (Dean, Harwood, Schechter)

B. Immunological studies of human hemoglobin ontogeny

Human fetal hemoglobin (HbF) differs from HbA in both functional and structural aspects. These differences are evidence by the weaker affinity of HbF for 2,3-diphosphoglyceric acid, by the non-participation of HbF in gel formation with HbS, and by the incomplete crossreactivity between antisera to HbF and other hemoglobins. We are using immunoabsorption with hemoglobin A to isolate non-precipitating antibodies specific for the δ chains of hemoglobin F which do not cross react with HbA_3 or other hemoglobins. A radioimmunoassay using reductively methylated ^{3}[H]-hemoglobin F, non-homologous hemoglobins and synthetic peptides from regions of the δ chains has been used to assess the specificity of binding. The antibodies

have been used to quantitate small amounts of HbF in standard laboratory procedures, in erythroid cell cultures and in prenatal diagnosis of hemoglobinopathies. (Eastlake-Dean, Schechter)

C. Hemoglobin synthesis in cultured erythroid cells

Fetal hemoglobin (or hemoglobin F) is the major hemoglobin produced during the later part of fetal life. The genes encoding for the gamma chain (the nonalpha chain of hemoglobin F) and the beta chain (the non-alpha chain of hemoglobin A) are in close proximity on chromosome 11. At birth, hemoglobin F is replaced by hemoglobin A as the predominant protein within the red cell. However, throughout life small levels of hemoglobin F persist in a small population of cells designated as F cells.

We are studying the expression of hemoglobin synthesis in cultures of erythroid cells. Depending on the source, several types of colonies designated colony forming units and burst forming units develop. Previous reports show the expression of hemoglobin F is induced in those colonies, the burst forming units, which take longer to develop and require higher levels of the hormone erythropoeitin. Using a sensitive and specific radioimmunoassay we will study hemoglobin F synthesis and its relation to time, culture conditions and source of culture material and then examine the molecular mechanisms related to the switch. (Luskey, Schechter)

D. Sickle cell anemia: deoxyhemoglobin S solubility and cell sickling studies

The gelation of deoxyhemoglobin S is believed to cause the sickling of SS erythrocytes. The solubility assay for characterizing a deoxyhemoglobin S gel was used to search for potential antisickling agents. We found that although several peptides from the hemoglobin sequence enhanced gelation, the β^{82-91} peptide could inhibit gelation of deoxyhemoglobin S to the same extent as phenylalanine and the β^{84-85} (Thr-Phe) peptide.

To study intracellular gelation, we developed an assay using ^{13}C double-resonance techniques. Data on samples of deoxyhemoglobin S gels obtained using ^{13}C NMR were correlated with data obtained using the sedimentation technique on the same samples. A correction to the deoxyhemoglobin S solubility obtained from sedimentation was necessary due to concentration gradients introduced during ultracentrifugation. Having thus found agreement between the two techniques, studies of intracellular-gelation as a function of oxygen pressure on SS erythrocytes was begun. (Noguchi, Schechter)

E. Sickle cell anemia: design and synthesis of gelation inhibitors

The regions of contact between deoxyhemoglobin S tetramers in the crystal form have been analyzed with a computer graphics system. Peptides complementary to two of these contact regions have been projected and their interactions with the hemoglobin analyzed visually. Several of these peptides have been synthesized by solid-phase and solution methods and purified and characterized. These peptides have been tested on the kinetic and solubility assays of deoxyhemoglobin S gelation. All, so far, have a negative effect, probably due to excluded volume. New peptides are being designed and synthesized using energy minimization and other considerations

to enhance their potential effectiveness as inhibitors of sickling.

To study in more detail the mechanism of excluded volume, we have tested a variety of short homopolymers of the most soluble amino acids. It has been found that lysine and serine peptides have the effect on activity coefficient predicted from their weight fraction; proline however, has a lesser effect, suggesting a specific molecular interaction. (Pavone, Eastlake-Dean, Schechter)

F. Location of the antigenic determinants on normal and sickle hemoglobin

We are using quantitative immunochemical methods to locate the antigenic determinants on hemoglobins A and S. We have changed the metal in the porphyrin of the heme group in both hemoglobins and are studying these reconstituted hemoglobins by quantitative precipitin and radioimmunoassay to see if any significant conformational changeshave been induced. These studies will allow us to detect subtle changes in the conformation of hemoglobins with zinc, copper, cobalt, manganese and nickel metalloporphyrins. Further, the metalloporphyrin and the chemical modification studies should allow us to locate antigenic determinants on the hemoglobin molecule. In addition we are studying the effects on the gelation of sickle hemoglobin of the metalloporphyrin substitutes. A solubility assay in buffers of high phosphate concentration is being used for this purpose. (Alston, Eastlake-Dean, Schechter)

IV. Studies on human interferon

A. Purification, chemical characterization of human interferon

Human lymphoblastoid interferon has been purified to a specific activity of 4×10^8 International Units/mg protein. The homogenity of this preparation has been suggested by the appearance of a single band on analytical SDS polyacrylimide gel electrophoresis, and by attainment of constant specific activity and amino acid composition after an additional purification step. A complete amino acid analysis of purified interferon analytical tryptic peptide maps and CNBr cleavage fragments of small amounts of high specific activity radiolabelled interferon have been obtained. Work is now in progress to determine the N-terminal amino acid sequence of lymphoblastoid interferon by LKB solid phase and Beckmann spinning cup sequenators. (Anfinsen, Bridgen, Miller, Smith, Zoon, Zur Nedden)

B. Purification and <u>in vitro</u> translation of human lymphoblastoid messenger RNA

Poly(A)-bearing RNA containing the interferon mRNA has been isolated from Namalva cells induced with Newcastle disease virus and translated in the <u>Xenopus laevis</u> oocyte system. The interferon mRNA has been purified 30-fold by sedimentation on a sucrose gradient under denaturing conditions with a 30% recovery and has a sedimentation value of 135 relative to globin mRNA. Using ^{35}S-methionine interferon of sufficient radioactivity has been obtained for initiation of amino acid sequencing studies. (Zoon)

C. Preparation of high specificity antisera to human interferon and their use in radioimmunoassays

Antibodies of high specificity toward human lymphoblastoid interferon have been produced in sheep and rabbits. These have been used for preparation of high capacity immunoadsorbant affinity chromatography columns for use in purification of interferon.

Antibodies to interferon have also been produced by in vitro cultures of lymphocyte hybridomas. Experiments are now in progress to clone the hybrid cell lines in soft agar and produce monoclonal IgG or IgM for use in radioimmunoassay and receptor studies.

Using ^{3}H methylated interferon as labeled antigen, standard curves for quantitative immunoprecipitation assays have been made. With the present specific radioactivity of labeled interferon the radioimmunoassay can be sensitive to within 10-100 Units of interferon or 10-fold less than present bio-assay.

V. Studies on protein components P. multimicronucleatum

A. Purification and characterization of the surface antigens (i-antigen) of Paramecium multimicronucleatum

When paramecia are placed in dilute homologous antisera, they become immobilized and eventually die. This reaction, the immobilization reaction, is the result of a specific interaction between homologous antibodies and the immobilization antigen (i-antigen) located on the surface of the cell. The i-antigen is distributed over 90 percent of the cell membrane. While a clonal population of cells has been shown to express only one antigen (serotype) at any given time, individual cells are capable of expressing upwards of twelve different antigens giving rise to the phenomenon of antigenic variation . A systematic phenomenon of antigenic variation has now been shown to exist in several species of protozoa including both the free-swimming forms such as paramecia and the parasitic forms, such as trypanosomes. (Steers, Barnett, Lee)

B. Purification and characterization of the myoglobins from paramecium

The distribution of heme-containing respiratory pigments capable of reversibly binding oxygen is well documented throughout the animal kingdom. Work in our laboratory has shown that at least two species of the ciliate protozoan Paramecium, contain such respiratory pigments. One of the species, P. tetraurelia has been shown to contain five electrophoretic variants. These multiple forms appear to be identical in molecular weight and amino acid composition and by spectral analysis although differing in their isoelectric points. The present project is concerned with an analysis of the functional aspects of each of the five molecular forms as well as attempts to isolate and characterize the myoglobin from a second ciliate species - P. multimicronucleatum. (Steers, Barnett, Lee)

C. Cell-cell interactions in protozoa. Isolation and characterization of mating-type substances (M-agglutins)

Conjugation (or mating) in Paramecium (as in numerous other species of protozoa and bacteria) occurs between complementary cells during special periods of reactivity. The interaction between reactive cells of opposite mating type shows an unusually high degree of specificity. When cells of opposite (complementary) mating type are mixed in culture, they adhere to one another on contact forming clumps containing as many as twenty cells. The initial agglutination of reactive cells involves only the cilia of the animals and only those cilia which are located along the oral surface. Within minutes of contact, the initial agglutination reaction is followed by pairing of complementary cells which separate from the initial clump. The complementary pairs undergo a fusion of their membranes along the oral surface which is followed by a reciprocal exchange of gametic nuclei and eventually mitosis. The process of specific cell agglutination and the subsequent events leading to mitosis are reminiscent of the biological actions associated with another class of agglutinus - the lectins. However, it now appears that the mating-type substances in paramecia are non-conjugated proteins. The project outlined in this report is designed to isolate and chemically characterize the substance(s) involved in the highly specific cell recognition and agglutination reaction referred to as mating. (Steers, Barnett, Lee)

VI. Protein factors controlling species-specific sperm-egg interaction

Immature mouse follicles are isolated and grown in culture for nine days and then biosynthetically labeled with radioactive amino acids or sugars. During this maturation period the zona pellucida an acellular matrix comprised of glycoproteins is formed around oocytes. The proteins of the oocyte and zona pellucida can be displayed on two-dimensional O'Farrell gels and followed for changes during this period of oocyte development. Methods are being developed to isolate the zona pellucida from the oocyte and determine its protein composition. These proteins are thought to mediate the specie-specific sperm-egg interaction by acting as recognition markers and subsequent sperm binding sites. We hope to identify and characterize these proteins both as to their physical properties and site of synthesis. (Dean, Harwood)

VII. Specific interaction of human C3 with erythrocytes and leukocytes

Interaction of the complement protein C3 with specific receptors present on leukocyte and lymphoid cells is important to the modulation of immune response and immune surveillance pathways. Our interests in these processes have been two-fold: 1) to measure the kinetic and equilibrium parameters of interaction between C3 and its several activation fragments with their receptors on human cells and 2) to identify the chemical mechanism by which C3 attaches to a target cell membrane. The activation fragments C3b, C3c, and C3d have been purified from limit proteolytic digests of human C3. C3 and C3d have been radiolabeled and specific binding to human erythrocytes and B lymphocytes measured by direct methods. The binding of either ligand was a saturable reaction with an apparent association constant of $1 \times 10^{7}\ M^{-1}$. Specific antibodies have been prepared to each of these fragments and have been used to develop sensitive radioimmunoassays. We are

presently investigating the possibility that a covalent bond between C3b and the membrane may be formed as a result of transesterification. We have detected a single sulfhydryl group upon either enzymatic conversion of C3 to C3b or treatment of C3 with hydroxylamine or hydrazine. It is our hypothesis that this sulfhydryl group is important to the activation of an acyl group within the C3 protein. (Tack, Lorenz, Schechter)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,000-06 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Purification, Chemical Characterization of Human Interferon

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.B. Anfinsen	Chief	LCB NIAMDD
	P.J. Bridgen	Visiting Fellow	LCB NIAMDD
	D. Miller	Chemist	LCB NIAMDD
	M. Smith	NIAID Fellow	LCB NIAMDD
	K. Zoon	Staff Fellow	LCB NIAMDD
	D. Zur Nedden	Bio-lab Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.4	3.6	0.8

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human lymphoblastoid interferon has been purified to a specific activity of 4×10^8 International Units/mg protein. The homogenity of this preparation has been suggested by the appearance of a single band on analytical SDS polyacrylimide gel electrophoresis, and by attainment of constant specific activity and amino acid composition after an additional purification step. A complete amino acid analysis of purified interferon analytical tryptic peptide maps and CNBr cleavage fragments of small amounts of high specific activity radiolabelled interferon have been obtained. Work is now in progress to determine the N-terminal amino acid sequence of lymphoblastoid interferon by LKB solid phase and Beckmann spinning cup sequenators.

PHS-6040
(Rev. 10-76)

Project Description:

Production: Standard production methods are now in regular use at the NIAMDD pilot plant and Fort Dietrick with total production at 0.5-1 x 10^9 International Units per month. Three 200 liter cultures of Namalwa cells are prepared monthly by the methods previously described by Zoon and Buckler (J. Clin. Micro. 7:44, 1978). Beginning 1 Sept. 1979 we will begin use of the large scale culture facilities at the Frederick plant which will enable the production of 1000 liter cultures every two to three weeks. Although some of this increased production is tentatively scheduled for use in clinical trials, the amount of crude interferon available for our purification will be increased to approximately 2.5 x 10^9 units per month.

Purification of human lymphoblastoid interferon: A general summary of the purification procedure is shown in the following table.

Purification of Human Lymphoblastoid Interferon[b]

Step	Units Recovered x 10^{-6}		Protein (mg)		Specific Activity (units/mg)		Recovery Range
	A	B	A	B	A	B	
Sephadex G-150	490	200	60.6	55	8.1×10^6	3.6×10^6	40-60
Ultrafiltration	440	200	60.6	55	7.3×10^6	3.6×10^6	90-100
SO-Sephadex C-25	296	-	20.1	-	1.5×10^7	-	65-100
Glycosidase Mixture Treatment	296	200	20.4	55.3	1.5×10^7	3.6×10^6	100
L-tryptophyl-L-tryptophan-Affi-gel 10	250	170	5.1	18.8	4.9×10^7	9.0×10^6	84-87
SDS PAGE[a]	49	35	.11	.14	4.5×10^8	2.5×10^8	27-31

[a]The amount of interferon recovered in the CBB-R250 component with the apparent molecular weight of 18,000.

[b]Total recovery of interferon activity from A, (7.8×10^8 units in TCA precipitates) to the peak fraction of the gel is approximately 6%. Total recovery from B (1.2×10^9 units in TCA precipitates) is approximately 3%.

The crude interferon is first purified by affinity immunoadsorbent chromatography, then by molecular weight separation on Sephadex. After gel filtration, a charge separation is performed on a small ion exchange column chromatography of SP-Sephadex. The partially purified material is then treated with a mixed glycosidase enzyme preparation which decreases the apparent molecular weight heterogenity in subsequent separation steps. An affinity column of Tryptophanyl-tryptophan bound through a spacer arm to agarose is then used to bind interferon by hydrophobic interactions. All concentration steps are done by ultrafiltration on millipore or amicon membranes with no loss of interferon activity. The final step is preparative polyacrylimide gel electrophoresis in the presence of SDS. The inter-

feron containing bands are eluted from the gel and this purified material is then used for amino acid composition (shown in the following table), chemical and enzymatic fragmentation and radiolabelling procedures.

Amino Acid Composition of Human Lymphoblastoid Interferon[a]

Amino Acid	Residues/155 Amino Acids Residues[b] (X + s.d.)[c]
Asx	16.3 ± 0.7
Thr	7.8 ± 0.6
Ser	11.0 ± 1.4
Glx	24.6 ± 1.9
Pro	N.D.[d]
Gly	N.I.[e]
Ala	10.4 ± 1.5
Cys	N.D.
Val	9.1 ± 1.4
Met	1.6 ± 0.8
Ile	7.4 ± 0.4
Leu	16.0 ± 1.2
Tyr	4.2 ± 1.3
Phe	8.1 ± 1.2
His	N.D.
Lys	13.2 ± 1.2
Arg	10.5 ± 1.2
Trp	N.D.

[a] Amino acid analyses were done at the National Geophysical Laboratories, Washington, D.C. by Dr. P.E. Hare using high-performance liquid chromatography of o-pthalaldehyde derivatives of amino acid residues and a fluorescence detection system. (Methods of Enzymology, 47, 2-18, 1977.

[b] Based on the molecular weight of 18,500, the number of amino acid residues in interferon is 155.

[c] The mean of 7 determinations of the amino acid composition of interferon eluted from SDS gels.

[d] Not determined.

[e] Not integrated.

Radiolabelling of lymphoblastoid interferon: Highly purified interferon fractions have been labelled with several radioactive indicator molecules. Antiviral activity was retained after reaction with 3H dansyl chloride and the labelled protein co-migrated with the peak of antiviral activity on SDS polyacrylimide gel electrophoresis. Purified interferon fractions were also labelled with ^{125}I by incubation with Bio-Rad enzymobeads containing lactoperoxidase and glucose oxidase. This iodionated material

retained 90-100% of antiviral activity and also co-migrated with the band of interferon activity on analytical SDS polyacrylimide gel electrophoresis. Purified interferon fractions were labelled by reductive methylation using formaldehyde and NaB_3H_4. After this reaction no measureable antiviral activity was detected.

Structural studies have been initiated using the iodionated interferon for generating cyanogen bromide fragments. Our amino acid analysis shows 2 methionines per molecule. Preliminary results show a very small change in the apparent molecular weight after cleavage indicating the presence of CNBr sensitive methionine residues near either end of the protein chain. Similar cleavage products from 3H methylated interferon also shows only slight decreases in molecular weight confirming the location of methionines. Tryptic fragments of the iodionated interferon have been prepared and two dimensional peptide map analysis has been performed to give the characteristics of the tyrosine containing peptides. These maps are also being used to qualitatively compare different highly purified interferon purifications for consistency in pattern of tryptic peptides.

Sequencing of Human Lymphoblastoid Interferon: Following the purification of lymphoblastoid interferon, the minute amounts which have been isolated will be subjected to sequence analysis by an LKB solid phase sequencer using ^{35}S phenylisothiocyanate or a modified Beckmann spinning cup sequenator in collaboration with Drs. Hood and Hunkapillar at California Institute of Technology. For solid phase sequencing the protein is attached to a glass support using a phenylenediisothiocyanate coupling procedure. Native interferon has given only low coupling yields but these yields can be increased by reducing and carboxymethylating the molecule before attachment. Fractions from the sequencer are converted to the phenylthiohydantoin (PTH) residues. Identification of these residues is achieved by adding a mixture of standard PTH's to each fraction. This mixture can then be analyzed by high pressure liquid chromatography (HPLC) using a program which has been designed to separate and collect each PTH as a distinct fraction. These fractions can then be counted in a scientillation counter and the radioactive residue identified. Spinning cup sequencing is performed by use of analytical HPLC sensitive to subnanamole levels. The residues from each sequence itself are automatically converted to the PTH derivative and quantitated directly by absorbance on the HPLC.

To determine the complete sequence of the interferon molecule, the purified labeled interferon will be fragmented two different ways to produce overlapping peptides using: 1) trypsinization of succinylated interferon which produced COOH-terminal arginine peptides; and 2) cyanogen bromide treatment of the purified interferon, which results in the formation of COOH-terminal homoserine lactone peptides. These peptides will then be separated by chromatography and sequenced. By comparing the amino acid composition of the two sets of overlapping peptide fragments, the correct sequence can be obtained.

The large scale production, purification and sequencing of interferon will be continued and if successful, studies will be initiated to synthesize it and determine the antiviral activity and the rate of catabolism of this molecule compared to the control purified interferon.

Interferon Assays: The standard bio-assay for interferon activity is now being done in our laboratory. Previously all assays were performed by contract at Biofluids, Inc., Rockville, Md. The assay procedure (Armstrong Applied Micro 21 723, 1971) consists of micro titer test plates using human foreskin fibroblasts and vesicular stomatitis virus standardized against international reference standard (G-023-901-527) by semiquantitative determination of cytopathic effect. The assay takes approximately 48 hours to obtain results. We have preliminary data which indicates new procedures for shortening the time to 24 hours.

Publications:

Zoon, K., and Buckler, C.E.: Large scale production of human interferon in lymphoblastoid cells. Texas Reports Biol. Med. 35: 145, 1977.

Klein, F., Ricketts, R., Jones, W., DeArmon, I., Temple, M., Zoon, K., Bridgen, P.: Large scale production and concentration of human lymphoid interferon. Antimicrobial Agents and Chromotherapy 15: 420, 1979.

Zoon, K.C., Bridgen, P.J. and Smith, M.E.: Production of human lymphoblastoid interferon by namalwa cells cultured in serum-free media. J. General Virology, in press.

Zoon, K.D., Bridgen, P.J., Smith, M.E., Anfinsen, C.B. and Zur Nedden, D.: Purification of human lymphoblastoid interferon. Proceedings of the International Workshop on Interferon, Sloan Kettering Institute 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,001-05 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of the Surface Antigens (i-antigen) of Paramecium multimicronucleatum

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edward Steers, Jr.	Research Biologist	LCB NIAMDD
	Audrey Barnett	Guest Worker	LCB NIAMDD
	Clifford E. Lee	Physical Science Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.6	0.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

When paramecia are placed in dilute homologous antisera, they become immobilized and eventually die. This reaction, the immobilization reaction, is the result of a specific interaction between homologous antibodies and the immobilization antigen (i-antigen) located on the surface of the cell. The i-antigen is distributed over 90 percent of the cell membrane. While a clonal population of cells has been shown to express only one antigen (serotype) at any given time, individual cells are capable of expressing upwards of twelve different antigens giving rise to the phenomenon of antigenic variation. A systematic phenomenon of antigenic variation has now been shown to exist in several species of protozoa including both the free-swimming forms such as paramecia and the parasitic forms, such as trypanosomes.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: With the isolation and comparison of several i-antigens from Paramecium tetraurelia, a second series of studies was begun on the surface antigens of a second species of ciliate - P. multimicronucleatum.

Methods Employed: Paramecia are grown on a grass infusion medium inoculated with Klebsiella cloacae and maintained in 5 gallon carboys. Several carboys (8-10) are harvested by passing the culture fluid through a commercial cream separator followed by low-speed centrifugation in a clinical centrifuge. The packed cells are extracted and the resulting supernatent is fractionated by a combination of ammonium sulfate precipitation and ion-exchange chromatography. A final purification step is carried out using isoelectric focusing.

Purified i-antigen was studied using a variety of techniques including immunodiffusion, analytical ultracentrifugation and automated amino acid analyses. Antigen purified by the conventional procedures outlined above was used to raise specific immune sera in rabbits. Immunoaffinity columns were prepared for the simpler, larger scale purification of antigen as well as the separation of antigens which are nearly identical in physical-chemical properties but fail to cross-react immunologically.

Major Findings: The major i-antigen C from P. multimicronucleatum has been purified by immunoaffinity chromatography. In addition, a second i-antigen (B) is being prepared for comparison.

Proposed Course: Further characterization of the surface i-antigens from P. multimicronucleatum is proposed. These studies will include an immunological analysis of each antigen together with the standard physical chemical comparisons on the native and denatured molecules.

Publication:

Davis, R.H. Jr. and Steers, E. Jr.: Purification of the i-antigen 51A from Paramecium tetraurelia by immunoaffinity chromatography. Immunochemistry 15: 371-378, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,002-06 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of the Myoglobins from Paramecium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edward Steers, Jr.	Research Biologist	LCB NIAMDD
	Audrey Barnett	Guest Worker	LCB NIAMDD
	Clifford E. Lee	Physical Science Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.6	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The distribution of heme-containing respiratory pigments capable of reversibly binding oxygen is well documented throughout the animal kingdom. Work in our laboratory has shown that at least two species of the ciliate protozoan paramecium, contain such respiratory pigments. One of the species, P. tetraurelia has been shown to contain five electrophoretic variants. These multiple forms appear to be identical in molecular weight and amino acid composition and by spectral analysis although differing in their isoelectric points. The present project is concerned with an analysis of the functional aspects of each of the five molecular forms as well as attempts to isolate and characterize the myoglobin from a second ciliate species - P. multimicronucleatum.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The objective is to extend our previous studies on the myoglobins of P. tetraurelia to functional analyses using the methods of flash photolysis and stop-flow. In addition, to isolate and characterize the myoglobin forms in other ciliate species.

Methods Employed: Methods were adapted for large scale cultures of Paramecium on grass extracts in 5 gallon carboys. The harvested cells (usually 100 liters) were homogenized in a raffinose - phosphate buffer after which the supernatent was fractionated by ammonium sulfate precipitation. The myoglobin containing precipitate was further fractionated on Sephadex gel filtration for the heme-protein mixture.

A final step in purifying the individual heme-proteins required either eluting the separated heme-containing zones from polyacrylamide disc gels or, alternatively, fractionating the myoglobins by isoelectric focusing in a sucrose gradient over a narrow pH range (3.5-6.0).

The purified myoglobins were characterized for size by analytical ultracentrifugation and for amino acid composition on an automated analyzer. Antisera was prepared against a partially purified fraction (G-50 fraction) by subcutaneous injection into rabbits.

Double diffusion in agar was carried out between immune sera and myoglobin samples separated on PAG.

Major Findings: The molecular weight determinations on the isolated species showed no significant differences. The average molecular weight value calculated for all determinations by sedimentation equilibrium was 15,000 daltons. The amino acid composition of all fine molecular forms appears identical and was calculated to be lys_{12} his_{3} arg_{4} asp_{17} thr_{9} ser_{6} glu_{16} pro_{4} gly_{11} ala_{15} cys_{0} val_{8} met_{2} ile_{7} leu_{10} tyr_{5} phe_{7}. Surprisingly, while the five species appear to differ only in their isoelectric points of the several physical and chemical properties analyzed, they do not show complete identity immunologically. Preliminary comparisons with immune sera show the minor species and a major Mb-2 species, Mb-5, show partial identity with the three other species: Mb-1, Mb-3 and Mb-4, presumably reflecting conformational differences among at least two of the five forms.

Significance to Biomedical Research: Many invertebrate hemoglobins and myoglobins have been identified from a variety of sources. This is the first instance of a molecule of this class appearing in the protozoa. Of special interest is the possible role of this molecule (and its variant forms) which may differ from that ascribed to these pigments in higher organisms.

Proposed Course of Project: Studies are now underway in collaboration with Dr. Joseph Bonaventura of the Duke Marine Biology Laboratory on the functional aspects of oxygen binding to the paramecium myoglobins. In addition, comparative studies are underway using a second species of paramecium

Publication:

Steers, E., Jr. and Davis, R.H., Jr.: Purification and characterization of the myoglobins of Paramecium tetraurelia. Comp. Biochem. Physiol. 62B: 393-402, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,003-09 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

A Study of Peptide-protein Interactions Using a Model Semisynthetic RNase-S'

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	I.M. Chaiken	Research Chemist	LCB NIAMDD
	A. Komoriya	Visiting Fellow	LCB NIAMDD

COOPERATING UNITS (if any)

Department of Biochemistry, Duke Medical Center, Duke University, Durham, N. .

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Based on experimental and theoretical studies reported to date on RNase-S', it was postulated that the minimum structural information expressed in the N-terminal sequence of RNase-S is an α-helix on which the residues Phe and Met are specifically positioned for maximal apolar interactions with RNase (21-124). To test this postulate, a model dodecapeptide was previously designed and synthesized by solid phase method. Properties of this peptide in terms of its solution conformation and its affinity toward S-protein were characterized. Further, the ability of this peptide to help direct the regeneration of active RNase (21-124) tertiary structure, after the initial scrambling of the disulfide bonds, was determined. For crystallographic structural analysis, purified model sRNase-S' was successfully crystallized in the same space group as native complex. Efforts were made to crystallized synthetically derived native and model analogue peptides. At present, crystals of [Ala 13] sRNase (1-15) have been obtained. For a continuing study on RNase enzymatic mechanism, the characterization of the residual activity found for [4-F-His^{12}, des 16-20]sRNase-S' and covalent [4-F-His^{12}]sRNase-A is in progress.

Project Description:

Objectives: To show that it is not only possible to identify the role of a certain amino acid sequence as being structural, interaction recognition, and/or catalytic but also to design a functional peptide knowing the necessary functions of amino acid sequenc. To crystalize the purified model S-peptide: S-protein complex in the same space group as native for crystallographic determination of the peptide conformation in complex. Further, to crystalize s-peptide and model S-peptide analogues for the determination of their structure in absence of the interactions with S-protein. To design a model peptide:protein interaction system that allows the evaluation of the free energy contribution of the specific apolar interactions and the configurational entropy for further development of peptide modeling method. To characterize the nature of the residual activity (<.2% of RNase-A specific activity) observed for [4-F-His^{12}]sRNase-S'. To characterize the residual activity of the recently prepared covalent [4-F-His^{12}] sRNase-A (see the project report of G.A. Homandberg) for an ongoing study on RNase enzymatic action.

Methods: Solution conformations of the purified model peptide and native S-peptide were determined from their CD spectra in aqueous solution. In order to evaluate the α-helical propencity of the model peptide as compared to native S-peptide, CD spectra of the peptides were taken in varying amounts of α-helix inducing solvent trifluorethanol. The dissociation constants for the model peptide and native peptide were determined from the data on the activation of S-protein by the peptides. Using the procedure of Kato and Anfinsen, JBC 244, 1004 (1969), the ability of the above peptides for regenerating active tertiary structure of S-protein from the disulfide bond scrambled S-protein was determined.

The purified model S-peptide:S-protein complex was crystallized by the procedures of Chaiken, Taylor and Ammon JBC 252, 5599 (1977), with the following modifications. The protein and the final ammonia sulfate concentrations were lowered to 2.5 mg/ml and 25% respectively. Crystals of [Ala 13]sRNase (1-15) were crystallized from absolute methanol.

The residual activities found for the [4-F-His^{12}] analogues were, first, characterized with respect to the levels of RNase-A and -S contaminations. The enzymatic activity of the analogues in 40% dioxane with cCMP and UpA was used to estimate the level of RNase-S-like contaminant. In addition, a study state kinetic parameter, Km, associated with the residual activity of the analogues was determined.

Findings: Judging from the similarity of the CD spectra for the model peptide and native S-peptide, it would be reasonable to assume that the amounts of folded structures present in the peptides in solution are comparable. The α-helical propencity of the model peptide, consistent with our expectation, was found to be greater than that of native as judged by the ellipicity changes at 200 mμ as a function of trifluoroethanol content. The affinity of the model peptide was determined to be lower by a factor of 12 as compared to native S-peptide. When one considers the case of a single residue

replacement of Glu 9 by glycine where one order of magnitude decrease in the affinity is noted, a remarkable conservation of the affinity toward S-protein is apparent.

The present data suggest that the design of the model peptide retained the most of the essential interactions that stabilize the complex. A lower ability of the model peptide to regenerate active S-protein 3^6 structure observed poses a question to the above assertion, however. The flexibility of the model peptide as compared to native is expected to be greater, since all of the replaced residues in the model peptide has more than beta carbon atoms as their sidechains. The conformational space available to the residues with a beta carbon is known to be less than alanine or glycine. This greater flexibility of the model peptide may account for the observed lower affinity. Literature search was made to find an alternative amino acid to alanine that would provide α-helical conformation with less flexibility. α-aminoisobutylic acid seems to meet the above desired requirements.

In order to verify the presumed conformation of the model peptide in complex, the model peptide:s-protein complex was successfully crystallized. Preliminary x-ray diffraction analyses of the above crystals revealed that the space group of the above crystals is the same as native. The size of the crystals, however, at present is about 0.3 x 0.2 mm (the lower limit for x-ray analysis). In addition to the crystallization of the complex, we obtained a good indication that it would be possible to crystallize a linear dodeca peptide from methanol. The crystals of [Ala 13] RNase (1-15) were obtained. However, the crystals are not, as yet, well formed. One hopes that the crystals of peptides suitable for x-ray analysis be obtained. This presents an exciting opportunity not only to solve the α-helical structure to a resolution comparable to that of small molecules (<1Å) but also to determine the effects of interactions with S-protein on the conformation of the peptide in complex.

[4-F-His^{12}, des 16-20]SRNase-S' and [4-F-His^{12}]sRNAse-S' were found to be inactive (<.2% of RNase-A) at pH 7 and 5 with cCHP and UpA. The residual activity of these complexes was determined to be predominantly RNase-A like contaminant, since the measured Km for these derivatives are closely similar with the value for RNase-A. There was some indication of an increased relative activity of the [4-F-His^{12}] analogues at lower pH. In order to characterize the residual activity of the [4-F-His-12]SRNase-S' below pH 5 independent of acid-dissocation of the complex, the covalent [4-F-His^{12}]-SRNase-A was prepared enzymatically (see G.A. Homandberg's report on this subject). At present, we were able to show that the covalent form exhibited a greater residual activity, as compared to the complex analogues (1.1% versus 0.2% with cCMP at pH 7). Further detailed characterization of the residual activity for the covalent [4-F-His^{12}]SRNase-A are in progress.

Significance to Biomedical Research: The success of designing a highly active amino acid sequence based on rational analysis of the native S-peptide sequence suggest strongly that it would be possible to design a peptide that would have the specified property.

Proposed Course of Action: Preparation of larger model peptide complex

crystals for x-ray analysis and the well formed crystals of peptides. Preparation and characterization of the model peptide analogues with α-amino butylic acid replacing all alanine and a disulfide bond containing model peptide.

Publications:

Chaiken, I.M. and Komoriya, A.: Protein Semisynthesis. Trends in Biochemical Sciences 3: 269-271, 1978.

Komoriya, A., Krigbaum, W.R. and Chaiken, I.M.: On the Chemical Information Required in S-peptide for the Conformational Stabilization of Ribonuclease-S. In Siemion, I.Z. and Kupryszewski, G. (Eds.): Peptides 1978 - Proc. 15th Euro. Peptide Symp., in press.

Appendix to Z01 AM 25,003-09 LCB
October 1, 1978 to September 30, 1979
Contracted Project

Title: Crystallographic Analyses of Active Site Residue Conformations of [4-F-His^{12}] Analogues of Ribonuclease S and Model S-peptide: S-protein Complex Conformation

Investigators: H.C. Taylor Biochemist
David Richardson Biochemist Duke University
Jane Richardson Biochemist Duke University

Department of Biochemistry, Duke Medical Center
Duke University, Durham, N.C. 27705

Summary:

The crystal structure of [4-F-His^{12}, des16-20]sRNase-S' is now being refined using the computer graphic system, GRIP of University of North Carolina. A limited x-ray diffraction data has been collected for the crystals of [4-F-His^{12}] RNase-S' analogues with a RNase substrate, UpA diffused into crystal. Structure determination work on the model S-peptide: S-protein complex has been initiated. The preliminary diffraction data showed that the space group of this crystal is the same as native RNase-S' complex. Further, the crystals are amenable to a high resolution crystallographic analysis.

Project Description:

Objectives: To determine the extent of any induced conformational changes may be present in the active site of [4-F-His^{12}, des16-20]sRNase-S due to the fluorination of histidine 12. To obtain further refined structure of RNase-S for the structural comparison of native RNase-S with other semisynthetically derived RNase-S' complexes. To determine the conformation of the model S-peptide in complex for the verification of the rationales in the design of this model S-peptide.

Methods Employed: The crystals of appropriate complex are subjected to standard x-ray crystallographic analysis. The molecular graphic system, GRIP, will be utilized in model-building and refinement of structure.

Findings: The present electron density map of [4-F-His^{12}, des16-20]sRNase-S' generated using the calculated phases of refined RNase-S model or the phases from multiple isomorphous heavy atoms replacements data of Wychoff et al (1970) JBC 245, 305 suggest strongly the structural identity of the active site region for native RNase-S and [4-F-His^{12}, des16-20]sRNase-S'. Through model building procedure, the above analogue structure is being refined. The diffraction data on the crystals of the model S-peptide:S-protein complex showed that the space group of the complex is the same as

native. Hence, the determination of the model S-peptide:S-proteim complex conformation is now possible. The crystal structure of the complex will aid in the verification of the rationales in the design of the model S-peptide. Further, the crystal structure of the model S-peptide in complex should provide reasons for the observed diminished maximum activity (35% of RNase-S) for this model RNase-S analogue.

Proposed Course of Action: Proceed with the refinement of [4-F-His^{12}, des 16-20]sRNase-S using GRIP and refining the active site residue sidechains by deleting these atoms from the initial phase calculations. Continue collecting diffraction data on [4-F-His12, des16-20]sRNase-S' with UpA. Continue collecting diffraction data on the model S-peptide:S-protein complex.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,004-06 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Quantitative Affinity Chromatographic Studies of Interacting Macromolecular Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	I.M. Chaiken	Research Chemist	LCB NIAMDD
Other:	W.M. McCormick	Chemist	LCB NIAMDD

COOPERATING UNITS (if any)
D. Eilat, Hadassah Hospital - Hebrew Univ. of Herusalem, Israel
F. Veronese, Istituto di Chimica Farmaceutica dell' Universita di Padova
R. Bevilacqua, Istituto di Chimica Farmaceutica dell' Universita di Padova

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is part of a continuing program to develop and use bioaffinity chromatography to determine quantitative characteristics of macromolecule-involved interactions. A major study was completed on the quantitative expression in affinity chromatography of antibody polyvalency in antigen binding. Studies also were completed on the interaction of psychoactive drugs with the multisubunit enzyme, bovine glutamate dehydrogenase, and on the interaction of peptide ligands with bovine neurophysins.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To develop the theory and technology of quantitative affinity chromatography to include polyvalent protein-ligand systems.

2) To apply the technique to characterize the expression of bivalency in antibody recognition of antigens.

3) To study psychoactive drug interactions with the potentially multivalent bovine glutamate dehydrogenase by the quantitative chromatographic method.

4) To resolve the question of valency in the *in vivo* pituitary complexes of neurophysins with the peptide hormones oxytocin and vasopressin.

Methods Employed: Derivation and evaluation of new formulations for polyvalent systems were carried out, requiring desk calculation and least squares fitting routines provided by the MLAB program via the in-lab computer terminal.

For the studies on antibody interactions with antigen, various [phosphorylcholine]-Sepharose matrices were prepared and used in elution of radioisotopically labelled TEPC-15-derived bivalent IgA monomers and monovalent IgA Fab's. Equilibrium dialysis analyses for protein species to [^{14}C]phosphorylcholine served for obtaining control data.

Glutamate dehydrogenase-drug studies proceeded with synthesis of perphenazine-Sepharose and competitive elution studies with various butyrophenone and phenathiazine compounds.

A neurophysin-specific affinity matrix, Met-Tyr-Phe-ω-aminophenyl agarose, was designed and chemically prepared.

Major Findings: Studies have confirmed and utilized the situation that the quantitative affinity chromatographic method allows measurement of equilibrium constants for association of ligands with multivalent proteins.

Studies with the TEPC 15-phosphorylcholine system have shown that polyvalent antigen density has a profound effect on the apparent affinity of antibodies for antigens. The affinity chromatographic technique has been shown to be a potentially powerful tool for the direct measurement of microscopic equilibrium constants for antibody interactions with antigens.

The behavior of glutamate dehydrogenase on immobilized drug matrices has allowed the demonstration that both butyrophenone and phenathiazine substances bind at the same enzymic site, with affinities paralleling the enzymic inhibitory as well as pharmacological potencies of these drugs. Also the non-identical but interdependent relationship of drug binding sites and those for recognition of nucleotides and substrates has been delineated.

Met-Tyr-Phe-ω-aminohexyl-agarose was found to effect specific binding

to bovine neurophysins I and II. The potential for use of this matrix for studying hormone-neurophysin complexes was demonstrated.

It was resolved that the present technology of affinity chromatography on porous matrices does not allow unambiguous calculation of intrinsic rate constants. An estimate of the contribution of steric factors has been made. Rate constant applications must await development of uniformly accessible (probably nonporous) immobilized ligand systems.

Significance to Biomedical Research: In general, the increasing development of quantitative affinity chromatography will be of importance in allowing the elucidation of many kinds of interacting processes involving not only proteins but other macromolecular or even cellular systems. The approach should provide an alternative to several imprecise or impractical types of techniques now used to study complex, biologically important systems.

Proposed Course of Action:

1) To continue work on the use of the affinity chromatographic technique to characterize neurophysin-hormone and antibody-antigen interactions.

2) To continue to expand the use of quantitative affinity chromatography to study biologically important interactions.

Publication:

Eilat, D., and Chaiken, I.M.: Expression of Multivalency in the Affinity Chromatography of Antibodies. Biochemistry 18, 790, 1979.

Chaiken, I.M., Eilat, D., and McCormick, W.M.: Appendix: Derivation and Evaluation of Equations for Independent Bivalent Interacting Systems in Quantitative Affinity Chromatography. Biochemistry 18, 794, 1979.

Veronese, F.M., Bevilacqua, R., and Chaiken, I.M.: Drug-protein Interactions: Evaluation of the Binding of Antipsychotic Drugs with Glutamate Dehydrogenase by Quantitative Affinity Chromatography. Mol. Pharmacol. 15, 313, 1979.

Chaiken, I.M.: Quantiative Uses of Affinity Chromatography - a review. Anal. Biochem., in press.

Chaiken, I.M.: Preparative and Analytical Affinity Chromatography of Neurophysins on Methionyl-Tyrosyl-Phenylalanyl-Aminohexyl-Agarose. Anal. Biochem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,006-06 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Characterization of Bovine Neurophysin - Neurohypophyseal Hormone Complexes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.M. McCormick	Chemist	LCB NIAMDD
Other:	I.M. Chaiken	Research Chemist	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.1	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

As part of a continuing study of the neurophysin-peptide hormone system, the tripeptide analogue of vasopressin, L-methionyl-L-tyrosyl-L-phenylalaninamide, has been modified for use in photoaffinity labeling studies of the hormone binding site on neurophysin. The photo-sensitive peptide, L-methionyl-L-tyrosyl-p-azido-L-phenylalaninamide, was found by equilibrium dialysis in the absence of light to be reversibly bound to bovine neurophysin II. The dissociation constant, stoichiometry, pH-dependence and competition properties with hormone analogues indicate that the interaction is similar to the interaction of hormone with neurophysin. Covalent complexes resulting from irradiation were fractionated on an affinity matrix. Investigation of the specifically labeled complex which would not bind to the affinity matrix revealed a 1:1 stoichiometry of incorporation and when oxidized, digested with trypsin and subjected to peptide mapping, affinity label remained attached to the peptide mode up of residues 44-66. Details of the site of attachment within this peptide will be studied by sequencing techniques. Irradiation of photoaffinity label with neurophysins from other species produces similar complexes and demonstrates the general applicability of this system.

PHS-6040
(Rev. 10-76)

Project Description:

A major objective of this project is to study the interaction of hormone analogues with neurophysin in order to obtain information about the hormone binding site on the neurophysin molecule. The identification of amino acid residues of the protein involved in the peptide-protein binding interaction will tell much about the environment of the binding site and should yield important information about the 3-dimensional structure of the neurophysins.

A major technique being used is known as photoaffinity labeling. The photo label which has been designed for use in this study is L-methionyl-L-tyrosyl-p-azido-L-phenylalaninamide. This reagent has been found to bind to bovine neurophysin II reversibly and specifically in the dark, the manner of interaction being analogous to hormone binding by neurophysin. When photolyzed, 12-15% of the neurophysin forms a covalent complex with affinity label. A major portion of the covalently modified protein (60-80%) is unable to bind to an affinity matrix known to bind active neurophysin. An investigation of the amount of photoaffinity label incorporated in this inactive fraction shows 1 molecule of label incorporated per mole of protein. Peptide maps of oxidized and trypsin digested material indicate that the photoaffinity label is covalently attached within the peptide made up of residues 44-66. Sequencing to determine the exact site(s) of interaction is now being undertaken.

Comparative labeling studies have been done with bovine neurophysin I and the major neurophysins from human tissue. Common characteristics of these reactions seem to indicate some similarities in the binding sites of these proteins. Study of these covalent complexes, as is being pursued with bovine neurophysin II, will likely tell much of the details of the similarities, and also might indicate some subtle differences found in these binding sites. Other labels with the photoreactive group in different positions may increase the information obtained about the binding sites.

Information about peptide-protein interactions is important for understanding many biochemical interactions. We hope to learn what arrangement of residues is necessary to bring about the specificity of the binding interaction. Experimental proof is now beginning to support the existence of a precursor from which the neurophysins and hormones may be derived. Trapping the neurophysin in the bound state may help to explain the significance of this precursor molecule. It will be interesting to determine if the labeed neurophysin retains a conformation similar to the unlabeled or whether occupying the binding site will alter conformation significantly and thus allow the expression of many of the properties of storage, transport and release that have been studied.

No Publications

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,007-06 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Antibodies Against Neurophysin and Other Proteins and Their Use for Conformational and Biosynthetic Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	I.M. Chaiken	Research Chemist	LCB NIAMDD
Other:	J. Seeman	Biologist	LCB NIAMDD
	D.A. Sobieski	Chemist	LCB NIAMDD

COOPERATING UNITS (if any)

L.C. Giudice, CEB, NIAMDD
A. Fontana, University of Padova

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.3	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is part of a general program to use specific antibodies as tools to study the chemical basis and biosynthetic origins of native protein conformation.

Purified neurophysin antibodies have been used to identify biosynthetic precursors of neurophysins (and the closely associated peptide hormones oxytocin and vasopressin) which are produced in cell-free synthesis in the presence of bovine hypothalamic mRNA. The nature of recognition of neurophysins and precursors with the purified antibodies is being studied.

Antibodies prepared against thermolysin and two large fragments of this protein have been used in immunodiffusion assays and radioimmunoassays to study conformational properties of the native protein as well as the fragments.

Affinity chromatographic techniques have been used to isolate ribonuclease antibodies specific for the S-peptide region (residues 1-20). Competition radioimmunoassay experiments are being carried out to determine the precise specificity of the antibodies and to try to use these to design semisynthetic proteins with altered antigenicity.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:
1) To prepare antibodies specific for particular proteins.
2) To use these antibodies to study conformational properties of the native protein as well as protein derivatives and fragments.
3) To use neurophysin-specific antibodies to isolate proposed neurophysin biosynthetic precursor.

Methods Employed: For the neurophysin antibody studies, antisera previously were obtained by injection of appropriate antigen emulsions into rabbits, using the services of Meloy Laboratories for maintenance of rabbits, injection of antigen, and collection of sera. Specific antibodies are detected by immunodiffusion and radioimmunoassay; purification of these is effected by neurophysin-Sepharose affinity chromatography. Purified antibodies are used in conjunction with cell-free translation studies of neurophysin biosynthesis. Herein, wheat germ translations are performed with mRNA extracted from frozen bovine hypothalamic tissue, specific translation products are immunoprecipitated by antibodies, and the specifically-recognized proteins are compared with native neurophysins by peptide mapping.

For thermolysin and ribonuclease antibodies, the presence of specific antibodies is tested by immunodiffusion, radioimmunoassay, and, for the latter case, ribonuclease and S-peptide-Sepharose affinity chromatography. The radioimmunoassays also are used to test, by competition analysis, the conformational nature of particular protein fragments.

Major Findings: In the search for a postulated neurophysin precursor, a high molecular weight cross-reactant has been detected through immunoprecipitation from cell-free syntheses using hypothalamic mRNA. Peptide mapping has shown that the product obtained with anti-neurophysin II contains sequences analogous to those in authentic neurophysin II. The observations give chemical and immunological proof to the suggestion that the 10,000 mol. wt. neurophysins are synthesized in hypothalamic cells as parts of much larger precursors of up to 25,000 mol. wt..

The extent to which antibody recognition requires fully native conformation is being elucidated at present in the case of anti-neurophysins by radioimmunoassay with native neurophysin and several derivatives.

As indicated by immunodiffusion and radioimmunoassay experiments, a significant amount of cross-reactivity exists between thermolysin fragments and native but not denatured protein. The immunochemical data indicate that at least one of the fragments has the propensity to form a stable conformation in solution comparable to that which the corresponding region attains in native thermolysin.

Affinity chromatographic techniques have been used successfully to isolate antibodies specific for the S-peptide region of ribonuclease. Competition experiments indicate that these antibodies can be used to distinguish between native-like and non-native states and also to distinguish native-like proteins synthetically altered to reduce the extent of antigenicity.

Significance to Biomedical Research: It is hoped that immunological investigation of neurophysin structure will lead to articulation of the biosynthetic origins and conformational nature of neurophysins and of the peptide hormones, oxytocin and vasopressin that are transported by neurophysins in mammalian systems. Studies with ribonuclease and thermolysin antibodies are aimed at the general goal of understanding the relationship between primary sequence data and the attainment of protein conformation.

Proposed Course of Action:

1) Continued investigation of conformation and biosynthesis of neurophysin antibodies.

2) Extension of the thermolysin studies, by preparation of purified antibodies and their use to delineate the nature of thermolysin fragments as conformationally-stable folding domains.

3) To use the purified ribonuclease antibodies specific for the S-peptide region (residues 1-20) to study conformational parameters of native and synthetically altered protein species.

Publication:

Giudice, L.C., and Chaiken, I.M.: Immunological and Chemical Identification of a Neurophysin-containing Protein Coded by Messenger RNA from Bovine Hypothalamus. Proc. Natl. Acad. Sci. U.S.A., in press.

Vita, C., Fontana, A., Seeman, J.R., and Chaiken, I.M.: Conformational and Immunochemical Analysis of the Cyanogen Bromide Fragments of Thermolysin. Biochemistry, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,008-16 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Dynamics of Protein

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hiroshi Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD
	Diana S. Parker	Chemist	LCB NIAMDD
	Austine Davis	Chemist	LCB NIAMDD

COOPERATING UNITS (if any)
Laboratory of Molecular Biology, Molecular Structure Section, NIAMDD
University of Padova, Padova, Italy

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Conformation Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	0.7	1.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) As a ground work for the study of the relationship between the amino acid sequence and the stabilizing energy of the three dimensional structure, the X-ray diffraction of crystals of Nuclease-T bound with ligands has been measured at 2 to 4°C to resolution between 2 and 3A using a precession camera in order to determine the atomic coordinates. (2) On the basis of measurements using a stopped flow technique folding of nuclease A portion of nuclease B appears to be thermodyanamically and kinetically independent of the 19 residues at the NH_2-terminus. (3) The equilibrium constant of Nuclease-(1-126) with deoxythymidine-3',5'-diphosphate in the presence of 0.01M Ca^{2+} has been measured by a dialysis equilibrium method.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To understand the mode of interatomic interactions involving the three-dimensional structure of proteins which underlies the structure, function and evolution of proteins.

Working Hypothesis: Folding of the polypeptide chain of protein to the specific three-dimensional structure must involve a specific dynamic event which would not be observed in a polypeptide chain containing an arbitiary amino acid sequence. Since even transformation between two non-native conformations of a polypeptide chain containing say, more than 100 residues cannot occur by random search for all theoretically possible conformations within a realistic time and therefore some sort of pathway must be considered, the theoretical impossibility of random search for all possible conformations cannot be considered as unique for folding of the native conformation. It follows that this theory fails to predict whether or not a specific event leading to the formation of the unique three-dimensional structure occurs in the early phase of folding or whether the specific dynamic event is involved only in the late phase. Thus, these questions must be answered by experiments. As reported in previous years the studies of this section have indicated that a specific decrease in Gibbs free energy responsible for stabilization of the three dimensional structure occurs in the late phase of folding. Thus, a hypothesis is advanced that a global potential function may be operative in the three-dimensional structure to bind the polypeptide chain in the ordered state, (this global potential function may be not operative in the partially folded polypeptide chain) and that this global force may be manifested in the mutually strengthening cooperative interactions throughout the three-dimensional structure. On the basis of this hypothesis, the studies under this project are presently directed to understand the cooperative mode of the interatomic interactions.

Methods Employed and Major Findings: 1) The three-dimensional structure of Nuclease-T', formed by non-covalent interactions of two fragments Nuclease-T-(6-48) and Nuclease-T-(50-149), is assumed to involve a similar cooperative mode of interatomic interactions to that of intact nuclease. On the basis of this assumption, the fragment exchange technique has been developed in this section in order to assess the stabilizing energy of the three-dimensional structure of nuclease (see the previous report). In order to determine similarity of the three-dimensional structure of Nuclease-T to intact nuclease as a basis of this hypothesis the X-ray crystallographic study of Nuclease-T has been carried out (see the previous report). Using a precession camera, the X-ray diffraction of liganded Nuclease-T crystalls at 2 to 4°C (this temperature range must be strictly maintained) have been photographically measured at 17 planes through the reciprocal lattice. The sets of data providing approximately 4400 unique reflections (extending to 2.6A resolution and probably further) are satisfactorily fitted to each other after "merge". With the collaboration of Dr. Gerson Cohen, Molecular Structure Section, LMB, NIAMDD, these data will be processed in order to determine the atomic coordinates of liganded Nuclease-T at resolution between 2 and 3 Å, using

the phases created on the basis of the atomic coordinates of nuclease (determined at the Massachusetts Institute of Technology).

2) Successful chemical synthesis of one of the fragments of Nuclease-T with substituted residues would open the way to the exact determination of the relationship between the interactions involving individual residues and the hypothetical global force. As a ground work preparation of semisynthetic Nuclease-T for crystallization is continued with the collaboration of Dr. Carlo DiBello at the University of Padova. After the initial success of preparation of highly active semisynthetic Nuclease-T (see the report of the previous year), two repeat syntheses of Nuclease-T-(6-48) did not yield samples of equivalent quality as examined by partial purification (see the report of the previous year). Another synthesis of the fragment is currently underway at the University of Padova.

3) A nuclease A fragment containing 126 residues from the NH_2-terminus, Nuclease-(1-126) provides an excellent test object for direct examination of the requirement of folding of the entire polypeptide chain of nuclease for operation of the cooperative interaction (see the report of the previous year). Measurements of intrinsic viscosity, the Stokes radius of Nuclease-(1-126) in the presence and absence of pdTp (deoxy thymidine-3',5'-diphosphate) and Ca^{2+} and the values for V_{max}, Km and Ki (with pdTp) of the intrinsic enzymic activity of Nuclease-(1-126) have indicated that although Nuclease-(1-126) may form an active site similar to that of intact nuclease, the species bound with ligands (presumably at the active site) possesses a partially folded conformation - composed of ordered and flexible portions different from the compact structure of intact nuclease (see the report of the previous year). In order to determine the binding constant of pdTp to Nuclease-(1-126) in the presence of Ca^{2+} which is vital for interpretation of the results described above, Diana Parker has carried out the dialysis equilibrium experiment using an equipment of dialysis cell assembly made at National Institutes of Health. The results to the present time have given a tentative value for the association constant (2×10^4 M^{-1}) of pdTp in the presence of 0.01M Ca^{2+} at pH 8 at 20°C. This value supports the interpretation described above. More accurate determination of the association constant by dialysis equilibrium is now in progress, using ^3H-labelled pdTp.

4) Nuclease B, which contains an additional flexible amino acid sequence of 19 amino acid residues bound to the NH_2-terminus of nuclease A (see the report of the previous year) has been investigated in order to determine the influence of the extra residues on the refolding of the nuclease A portion from the acid dentured state by monitoring the change in tryptophan fluorescence using a stopped-flow technique. The kinetic parameters of this refolding are found to be similar within experimental errors for nuclease A and B for the entire time course (up to 40 sec) studied. Thus, the folding of the nuclease A portion of nuclease B appears to be thermodynamically and kinetically independent of the 19 residues at the NH_2-terminus.

5) A new study under this project has begun in order to measure cooperative interaction energy operating in the three-dimensional structure of protein. The idea is that application of the fragment exchange technique, in combination with chemical synthesis of a fragment to an ordered complex

formed from three or more fragments could provide important information concerning the cooperative interaction energy. Two three-fragment complexes are known. One is that of staphylococcal nuclease discovered in this laboratory (see the report in the early year) and the other of RNase A found at the Rockefeller University. The RNase A system is suitable for this investigation. As a start, Austine Davis has synthesized the COOH-terminal nonapeptide of RNase A by the established method (a solid-phase synthesis). The nonapeptide of approximately 50% purity was obtained after cleavage from the resin (the solid matrix), in a yield of approximately 50%. The sample deblocked and purified to homogeneity by ion exchange chromatography on SP-Sephadex C-25 generated the expected level of the enzymic activity with RNase-(1-118).

A portion of project, Mechanism of Renaturation of Reduced Lysozyme, #201 AM 25,009-04 LCB could not be reported in the report of the previous year: The products formed from hen egg lysozyme by incubation with 2 mM dithiothreitol at pH 7.8 23°C for 40 min. followed by reaction with [1-^{14}C]iodoacetic acid have been purified by gel ifltration and ion-exchange chromatography. An enzymically active derivative containing 4 mol of [1-^{14}C]carboxymethyl groups and no free sulfhydryl groups has been obtained in approximately 18% yield. Examinations of hydrodynamic volume, tryptophan fluorescence, CD and tryptic peptides containing [1-^{14}C] carboxymethyl cysteine has identified this species as the derivatives containing two, presumably native disulfide bonds and carboxymethyl groups at Cys 6, Cys 127, Cys 76 and Cys 94. The rest of the species in the incubation mixture are intact lysozyme. This two-disulfide bonded species is expected to be useful for investigation of thermodynamic stability of the two-disulfide bonded intermediate species in renaturation of reduced lysozyme.

Significance to Biomedical Research: The specificity of reactions and regulation of rate processes in cells is one of the fundamental characters of the biological system. An alteration of this control mechanism would result in malfunction of cells (a disease) unless this disorder is compensated by the system. One of the most important mechanisms of the specificity and the regulation is the specific function of globular proteins (e.g. enzymes, hemoglobins and immunoglobulins). This function of globular proteins is the work of interatomic interactions operating in the unique three-dimensional structure formed. However, the mode of operation of this interatomic interaction is not well understood. Thus, understanding the operational mode of interatomic interactions in the three-dimensional structure of proteins (the mechanism of self-ordering of the polypeptide chain in relation to the amino acid sequence) is fundamental to Biomedical Science.

Proposed Course: 1) Determination of the atomic coordinates of Nuclease-T. 2) Crystallization and X-ray diffraction studies of semisynthetic Nuclease-T' having the native sequence and analogues having substituted residues. 3) Development of a fragment exchange system of a three-fragment complex of RNase A in order to measure the cooperative interaction energy as a function of substituted residues using the fragment exchange technique and chemical synthesis. 4) Measurements of activation energy parameters of

unfolding of Nuclease-T as a function of concentration of guanidine HCl and pH using the fragment exchange technique in order to investigate the properties of the energy barrier. 5) Development of a physico-chemical method for testing the hypothetical global potential function to bind the polypeptide chain in the ordered state.

Publication: Davis, A., Parr, G.R. and Taniuchi, H.: A Kinetic Study of the Folding of Nuclease B, A Possible Precursor of Staphylococcal Nuclease A. Biochem. Biophys. Acta. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,009-05 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mechanism of Renaturation of Reduced Lysozyme

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.S. Acharya	Visiting Associate	LCB NIAMDD
	H. Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Conformation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Combined with Z01 AM 25,008-16 LCB

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,011-05 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Conformational Dynamics of Cytochrome c Using a Three-fragment Complex

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Juillerat	Visiting Fellow	LCB NIAMDD
	H. Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Conformation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to study the forces maintaining the conformation of cytochrome c, a biologically active, three-fragment complex composed of heme fragment (1-25)H, and apofragments (28-38) and (56-104) was developed. This complex was obtained by a limited tryptic digestion of the parent complex formed by heme fragment (1-25)H and apofragments (1-65) and (39-104).

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Studying the dynamics and structure-function of protein molecules using the three fragment complex system of cytochrome c.

Methods Employed and Major Findings: Preceeding studies in this laboratory (R.R. Hantgan, H. Taniuchi, J.B.C. 252, (1977), 1367-1374; G.R. Parr, R.R. Hantgan, H. Taniuchi, J.B.C. (1978), 5381-5388) showed that fragments of cytochrome c could form by non covalent bonding an active and ordered complex. Heme fragment (1-25)H and apofragment (23-104) as well as (1-38)H and (39-104) or (56-104) yield to fully active or productive complexes, that means the complex is reducible by ascorbate and L-lactate dehydrogenase, shows the characteristic 695 nm band indicating methionine ligation, and finally presents a CD and visible spectra similar to cytochrome c. Since in contrast, heme fragment (1-25)H and apofragment (39-104) has still some interaction but lead only to a non productive complex, it was natural to add the missing portion of the sequence by adding a third fragment. Addition of heme fragment (1-25)H to apofragment (39-104) and (1-65) results in a complex reducible by ascorbate and cytochrome b_5. Apoprotein (1-104), apofragment (39-104) heme fragment (1-25)H were prepared in the same way than described last year.

The heme fragment (1-38)H was prepared by modification of previous methods. Horse heart cytochrome was dissolved in water and solid guanidine hydrochlorine was added to yield a 6M solution. The pH was adjusted to 8.4 with 2N KOH. A 100 fold excess of citracomcanhydride was then added in 4 hours and the reaction mixture stands at RT for another two hours. After extensive dialysis at pH 9, the pH was set to 8.2 and the chemically modified cytochrome was cleaved by trypsin, the fragment were separated by gel filtration on Sephadex G50 in ammonium bicarbonate pH 8.3. Lysines were deblocked in 30% acetic acid at 4°C. The heme containing fragment was finally purified by gel filtration on Sephadex G50 in 10% formic acid and by ion exchange chromatography on SP-Sephadex C-25.

Apocytochrome c was dissolved in 70% formic acid and a 65 fold molar excess of cyanogen bromide was added. The reaction took 12 hours at RT. The sample was lyophilised and the resulting fragments separated by gel filtration on G50 fine in 10% formic acid. No further purification was necessary as shown by amino acid analyses.

A equimolar amount of fragment (1-25)H, (1-65) and (39-104) probably dissolved in 0.1M ammonium acetate pH 7.0 were mixed together and lyophilised. The lyophisat was dissolved again in the same buffer to a protein concentration of 6 mg/ml. The solution was then deaerated by a freeze thaw procedure and finally equilibrated with argon A50 fold molar excess of sodium ascorbate was added and after fifteen minutes a 1% trypsin solution was added (ratio enzyme-substrate = 1:1000). The digestion took place for 70 min. The resulting complex was isolated and purified by gel filtration on Sephadex G75 and ion exchange chromatography on SP-Sephadex C-25. The constituting fragments were analysed after separation on Sephadex G50 in 10% formic acid and were shown to be heme fragment (1-25)H and apofragment (28-38) and (56-104).

The parent complex (complex A = (1-25)H*(1-65)*(39-104)) as well as the complex reconstituted from fragments (1-25)H*(28-38) and (56-104) (complex B) were fully reduced by ascorbate. Biological activity was determined by comparing the rate of reduction of cytochrome c and complexes by yeast lactate dehydrogenase. Briefly, to 800 µl of 10^{-5}M complex or cytochrome c in 0.1M potassium phosphate, 25µl of yeast L-(+)-lactate dehydrogenase was added. The reduction was started by adding 10 µl of a 1M sodium L-(+)-lactate solution (pH 7.0). The reaction was monitored at 415 nm. Complex A showed 31% of an activity compared to native cytochrome c and reconstituted complex B still 18%.

Both complexes, showed the characteristic Soret band 406 nm in the ferric state, band which is shifted to 415 nm in the ferrous form. A 695 nm band in the ferric form, indicates the methionine-80 ligation.

The CD spectra in the 200-250 nm range shows an organized structure close to native cytochrome c.

Since only the three fragment complex is reducable by ascorbate, titration of the heme fragment plus one apofragment with the second apofragment in presence of ascorbate indicate a ratio of 1:1:1 of each constituting fragment. Amino acid composition agrees with titration data.

Titration of the ferric heme fragment shows dissociation constants of 7.6×10^{-7}M and 10^{-5}M. In reduced form they even reached values of less than 10^{-8}M and 10^{-7}M respectively.

The formation of a biologically active, non-covalent associating system involving three fragments derived from horse heart cytochrome c was achieved. This complex seems to be a suitable model for analyses of interatomic interactions by the fragment exchange technique.

Significance to Biomedical Research: The operational mode of interatomic interactions stabilizing the ordered structure of proteins are not well understood. Since the ordered structure of proteins is of prime importance for the biological function, understanding the mode of interatomic interactions involving the amino acid sequence folded to the three-dimensional structure is fundamental in biomedical research. This biologically active, three fragment complex of cytochrome c, now established, is expected to give an excellent test object to investigate whether a global potential function is operative in the ordered structure in order to bind the polypeptide chain. For measurements of the fragment exchange rate would give an unequivocal answer to the question as to whether the binding energy of each fragment is derived from the same global cooperative interaction. Since one of the three fragments, (28-38) can be readily synthesized, the system can also be used for the study of the effect of substitution of residues on dynamics and structure-function of cytochrome c and would help in understanding the reason why the specific residues are evolutionary invariant.

Proposed Course of Research: Fragment labeling for exchange studies, synthesis of the labeled fragment (28-38), exchange studies in order to test the binding energy between each fragment, and substitutions of several residues in fragment (28-38) by peptide synthesis in order to understand the hydrophobic effects of the heme moiety.

No Publications

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,013-08 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Hemoglobin Oxygen Affinity: Immunochemical Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. Dean — Senior Investigator — LCB NIAMDD
S. Harwood — Physical Science Technician — LCB NIAMDD
A.N. Schechter — Chief, Section on Macromolecular Biology — LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.5	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using non-precipitating monospecific antibodies we have been studying the conformations of hemoglobins in solution. Hemoglobin A_o and A_2 have been isolated, purified and isotopically labeled either by carbamoylation with $K^{14}CNO$ or by reductive alkylation with tritiated borohydride. A specific subpopulation of anti-hemoglobin antibodies has been isolated by affinity chromatography using a synthetic peptide corresponding to α(129-141) and has been used in a radioimmunoassay to study conformational changes that occur at the carboxy-terminus of α chains during oxygenation. These antibodies which bind with an affinity of $5x10^8\ M^{-1}$ to CO-liganded ^{14}[C] hemoglobin increase the oxygen affinity of hemoglobin and, thus, act as a linked function. A second subpopulation of antibodies has been isolated that react specifically with ^{3}[H]-hemoglobin A_2. These antibodies will be used in a radioimmunoassay to detect hemoglobin A_2 levels in serum and cultured erythropoietic cells as well as to differentiate A_2 from other normal and mutant hemoglobins.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: This laboratory continues to maintain its strong interest in the use of monospecific antibodies as probes of protein conformation and structure. We have recently isolated two specific antibody populations by affinity chromatography. One recognizes changes in the conformation of hemoglobin A_0 due to a difference in the tertiary structure between the T state (deoxy) and the R state (oxy). The other recognizes a difference in the conformation of hemoglobin A_2 and A_0 based on the primary sequence of the protein. These latter antibodies can differentiate hemoglobin A_2 from closely homologous hemoglobin species, and will be used to follow in vitro hemoglobin synthesis.

Methods and Major Results:

Hemoglobin A_0

Hemoglobin A_0 has been chromatographically purified on DEAE Sephadex and carbamoylated with K^{14} CNO. The extent of modification has been determined independently by hydantoin analysis and the quantitation of homocitrulline as well as by specific activity. Although structurally similar to unmodified hemoglobin, the carbamoylated protein does have a slightly higher oxygen affinity.

Sheep immunized with liganded hemoglobin A_0 have produced antisera which discriminates poorly between R (oxy) and T (deoxy) forms of hemoglobin when analyzed by quantitative precipitin curves or by a double antibody radioimmunoassay. To improve the ability of the antisera to differentiate between the two states we have used affinity chromatography to isolate a subpopulation of antibodies that are specific for the carboxyl terminus of the α chain of hemoglobin. It is in this region of the chain that the penultimate tyrosine and carboxyl-terminal arginine residues become exposed to the solvent in the oxy (R) state (having been protected in the deoxy (T) conformation).

To isolate such a monospecific subpopulation of antibodies we synthesized a tridecapaptide, α(129-141), analogous to the carboxyl terminus of the α chain by the Corley modification of Merrifield solid phase synthesis. This peptide was used to prepare a Sepharose-peptide affinity column that isolated from anti-hemoglobin antisera a protein peak that was demonstrated to contain immunoglobins by immunoelectrophoresis. Fluorescence quenching studies with these isolated antibodies showed the binding of a single antibody molecule to each hemoglobin dimer at a given time suggesting a marked restriction in the heterogeneity of the antibodies compared to the whole antiserum.

These monospecific antibodies designated α(129-141) anti-hemoglobin antibodies were then used in a direct binding assay with liganded and deoxy ^{14}C carbamoylated hemoglobin. The bound hemoglobin was separated from the free hemoglobin by a precipitating second antibody (burro-anti-sheep IgG) and the liganded state under assay conditions was confirmed spectrophotometrically. Particular care was needed in deoxygenating the dilute hemoglobin solution in order to prevent the formation of methemoglobin.

The α(129-141) anti hemoglobin antibodies showed a clear difference in their reactivity with CO and deoxy ^{14}C carbamoylated hemoglobin. They bound to the liganded hemoglobin with an affinity of $5 \times 10^8 M^{-1}$ and bound to deoxyhemoglobin with an affinity of 1×10^8 M^{-1} or less. We interpret the decreased binding to deoxyhemoglobin to represent the comparative inaccessibility of the carboxyl terminus of the α chain in the T (deoxy) state. Although presumably restricted in its heterogeneity, this antibody subpopulation interacts with [^{14}C]carbamoylated hemoglobin non-linearly on a Scatchard plot which suggests that there are several monoclonal antibody populations that react with the carboxyl terminus of the α chain.

The observation that α(129-141) anti-hemoglobin antibodies react preferentially with carboxyhemoglobin suggests that they should stabilize the liganded conformation and, therefore, increase the oxygen affinity of the hemoglobin. Experimentally the presence of these antibodies increased the P_{50} of normal hemoglobin about 20% with an apparent change in the heme-heme interaction. This second phenomenon, observed in a biphasic Hill plot, can be explained either by the presence of a hemoglobin solution incompletely saturated with antibody or by heterogeneity in antibody binding affinities. The lack of any further perturbation upon the doubling of the α(129-141) anti-hemoglobin antibody concentration argues against, although it does not exclude, the first explanation. (Further increases in antibody concentration were not possible because of problems of solubility.) The second possibility is supported by the nonlinear Scatchard plot.

This type of linked reciprocal binding (i.e. antibody and oxygen) has been observed with a number of heterotropic effectors that bind preferentially to liganded hemoglobin and the thermodynamic basis of this phenomenon has been carefully detailed and quantitated by Wyman. In this construct the affinity for oxygen is changed by heterotropic effectors, such as α(129-141) anti-hemoglobin antibodies, which bind a portion of the hemoglobin that is structurally distinct from the oxygen binding site. Regardless of the mechanism of interaction such binding can be analyzed thermodynamically by the introduction of the concept of median ligand activity, P_m. This term represents the PO_2 at which the concentrations of unliganded and fully liganded hemoglobin are equal and at that point is equal to the reciprocal of the K_{assoc} of hemoglobin for oxygen. The median ligand activity takes into account any asymmetry of a Hill plot and the free energy of interactions can be calculated by $\Delta G = tRT \ln P_m$, where t is the number of oxygen binding sites.

At equilibrium in this schema, the difference in the apparent free energies of oxygen interacting with hemoglobin tetramers in the presence and absence of monospecific antibodies should be matched by the difference between the free energies of antibody binding to liganded hemoglobin vs that of binding to deoxyhemoglobin. The first two free energies can be calculated directly from the oxygen affinity curves as described above and the latter two can be ascertained from the experimentally determined binding affinities of α(129-141) anti-hemoglobin antibodies to deoxy- and liganded hemoglobin dimers. If we assume that the change of free energy of one antibody molecule binding to one antigenic determinant is the same whether

the antigenic determinant is a part of hemoglobin dimer or of a hemoglobin tetramer and that the binding constants of antibody for both oxy- and carboxyhemoglobin are similar, then for two antibody molecules binding to the intact hemoglobin tetramer, $\Delta G = -2RT \ln K_{assoc}$.

Thus, if ΔG of oxygen bindings is -27.0 kcal/mole of hemoglobin tetramer in the absence of antibody and -27.9 kcal/mole of hemoglobin tetramer in the presence of antibody, then the difference between the two apparent free energies of interaction of oxygenation is 0.9 kcal/mole of hemoglobin tetramer. However, the ΔG using median association constants of antibody binding to liganded and unliganded hemoglobin is -21.7 kcal and -20.00 kcal/mole of hemoglobin tetramer respectively. Therefore, the difference in the free energies of antibody binding to liganded and deoxyhemoglobin is 1.7 kcal/mole of hemoglobin tetramer or a difference 0.8 kcal greater than we anticipated. This second quantity is a minimum difference calculated by assuming that the association constant of $\alpha(129-141)$ anti-hemoglobin antibodies with deoxyhemoglobin is approximately the same for all deoxyhemoglobin molecules, even though the radioimmunoassay is sensitive enough to measure the binding to only 15-18% as much deoxyhemoglobin as liganded hemoglobin. The several explanations of this apparent discrepancy in the differences in free energies are discussed in a recent publication but in any event antibody and oxygen clearly act as a linked function in binding to hemoglobin.

Hemoglobin A_2

An anlogous strategy was attempted in preparing a specific assay for hemoglobin A_2. Whole antisera was obtained from sheep immunized with hemoglobin A_2 and this sera, as determined by quantitative precipitin curves, could partially differentiate among A_2, A_0 and F hemoglobins. Hemoglobin A_2 was tritiated to a specific activity of 1×10^5 dpm/M of heme protein by alkylating it with formaldehyde to form a Schiff's base which was then reduced with tritiated borohydride. A double antibody radioimmunoassay was used to analyze the specificity and sensitivity of the antisera. Although there was a quantitative difference in the ability of unlabeled hemoglobins A_0, F and A_2 to displace tritiated hemoglobin A_2 from the antisera, there was some overlap among these species. Therefore, a synthetic peptide representing residues (5-13) of the delta chain of hemoglobin A_2, was used as an immunoabsorbant in an attempt to isolate monospecific anti-hemoglobin A_2 antibodies. Although the specificity of a subsequently developed radioimmunoassay was improved, it was still not optimum, and so hemoglobin A_0 was attached to Sepharose and used as an immunoabsorbant. The initial flow thru peak contained antibodies that could detect hemoglobin A_2 with specificity and sensitivity even in the presence of other homologous hemoglobin species.

Significance to Biomedical Research and Proposed Course of Project:
Elegant studies by Perutz and his colleagues in the 1960's have shown significant tertiary and quarternary changes in the conformation of hemoglobin upon oxygenation. We hope that an assay using monospecific antibodies to liganded hemoglobin in solution will complement these crystallographic studies and give further information about the dynamics of this conformational

shift. These antibodies could also be used to look at mutant hemoglobins with abnormal oxygen affinity in order to better define structure-function relationships.

Antibodies specific to hemoglobin A_2 will have direct clinical application in measuring levels of A_2 in the fetus and in the adult, as well as distinguishing A_2 from other hemoglobins which have a similar electrophoretic mobility (such as hemoglobin C). We also will use this assay to quantitate hemoglobin in cultured erythropoietic cells in order to examine at the molecular level the synthesis of hemoglobin A_2.

Publications:

Dean, J. and Schechter, A.N.: Sickle Cell Anemia: Molecular and Cellular Bases to Therapeutic Approaches, (Medical Progress) New England Journal of Medicine 299: 752-763, 804-811, 863-870, 1978.

Dean, J. and Schechter, A.N.: Conformation-Specific Antibodies to the α Chain Carboxyl Terminus of Hemoglobin A_o. J. Biol. Chem, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,014-05 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Functional Properties of Immune Response Gene Regulation of Antibody Response to Nuclease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A.N. Schechter Chief, Section on Macromolecular Biology LCB NIAMDD

COOPERATING UNITS (if any)
Immunology Branch, NCI (Dr. D.H. Sachs); Metabolism Branch, NCI (Dr. J. Berzofsky); Laboratory of Immunology, NIAID (Dr. R.H. Schwartz)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,016-06 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immunological Studies of Human Hemoglobin Ontogeny

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Eastlake-Dean	Chemist	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)
Unite de Recherche sur les Anemies, Hospital Henri-Mondor, Creteil, France (Drs. J. Rosa and Y. Beuzard).
Dept. of Med., School of Med., U. Washington, Seattle, WA (Dr. G. Stamatoyannopoulos)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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0.8	0.2	0.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human fetal hemoglobin (HbF) differs from HbA in both functional and structural aspects. These differences are evidence by the weaker affinity of HbF for 2,3-diphosphoglyceric acid, by the non-participation of HbF in gel formation with HbS, and by the incomplete crossreactivity between antisera to HbF and other hemoglobins. We are using immunoabsorption with hemoglobin A to isolate non-precipitating antibodies specific for the δ chains of hemoglobin F which do not cross react with HbA or other hemoglobins. A radioimmunoassay using reductively methylated ^{3}[H]-hemoglobin F, non-homologous hemoglobins and synthetic peptides from regions of the δ chains has been used to assess the specificity of binding. The antibodies have been used to quantitate small amounts of HbF in standard laboratory procedures, in erythroid cell cultures and in prenatal diagnosis of hemoglobinopathies.

Project Description:

Objectives: Immunologic studies in our laboratory of staphylococcal nuclease and, subsequently, of human hemoglobin, have used several new approaches. First, antibodies directed against specific regions of the amino acid sequence of these proteins have been isolated. Such antibody populations are sensitive to protein conformation in the region of their specificity. Second, radioimmunoassays have been used to demonstrate the specificity of the binding of such restricted antibody populations and to quantitate the ability of proteins fragments (especially synthetic peptides) representing the region of antibody specificity to compete in this specific interaction. In addition, the cross reactivity of closely related proteins may be quantitated by these methods.

Human fetal hemoglobin has 39 amino acid differences from HbA. Some of these substitutions result in antigenic determinants unique to each of these hemoglobins. The goal of this project is the isolation of antibodies specific for HbF and HbA and their use to quantitate precisely very small amounts of fetal and adult hemoglobins in research and clinical situations by the technique of radioimmunoassay. In particular we are following the levels of these hemoglobins in individual bursts of erythroid cells grown in culture with respect to time in culture, erythropoietin concentration and other factors. Such data should be useful in understanding the mechanisms related to differentiation, proliferation and globin gene activity in erythroid cells in culture.

Methods: The isolation of human HbF from umbilical cord blood samples and sickle cell anemia patients with high levels of HbF has been carried out by standard ion-exchange chromatographic procedures and analyzed for purity by isoelectric focusing in polyacrylamide slab gels. HbF was reductively methylated with ^{3}[H] sodium borohydride. Hemoglobin A was purified from bank blood and labelled by similar methods.

HbA was coupled to Sepharose beads by CNBr activation. Antisera to HbF was raised in sheep. A HbA-Sepharose conjugate was used to absorb all of the sheep anti-HbF antibodies capable of cross reacting with HbA. The specificity of this procedure was quantitated in a radioimmunoassay using 3[H]-HbF. Similar absorption and analysis procedures have been carried out to develop radioimmunoassays for HbA and HbS. Peptides have been prepared using a rapid modification of the Merrifield solid-phase method and purified by gel filtration and ion-exchange chromatography.

Cultures of erythroid precursors isolated from human umbilical cord blood and adult peripheral blood have been established using the methylcellulose system. Individual bursts have been lifted from culture dishes using ultrafine capillaries. Bursts have been lysed by freeze-thawing in water and assayed in radioimmunoassays for content of HbF and HbA.

Major Results: Highly purified HbF has been isolated from cord blood samples. HbF has been tritiated to high specific activity by reduction with ^{3}[H]-sodium borohydride (greater than 10^{9} DPM/mg specific activity).

Antibodies specific for HbF have been isolated by immunoabsorption with Hb. In a highly sensitive radioimmunoassay these antibodies were found to bind to HbF with an affinity constant of about $10^8 M^{-1}$ and measure HbF in the picogram range. Cross reactivity with HbA is not seen until concentrations four orders of magnitude higher are used which defines the broad concentration range over which the antibodies are totally specific for HbF. Accurate determination of the amount of HbF present is possible even when it represents only 1 part in 10,000 of the total hemoglobin in the sample. Preliminary experiments indicate it will be possible by this method to quantitate the amount of HbF present in samples obtained from fetuses for the antenatal diagnosis of hemoglobinopathies. A specific radioimmunoassay for HbA has also been developed for use in such studies.

Peptides representing specific regions of the HbF molecule have been synthesized. The radioimmunoassay is suitable for assessing the ability of such peptides to displace ^{3}[H]-HbF from the specific antibodies, thus, indicating their contribution as antigenic determinants for HbF. Preliminary results indicate that the peptide δ (68-81) of HbF has some weak but measurable ability to displace ^{3}H-HbF from the specific anti-HbF antibodies. This peptide sequence has 7 amino acid differences from the corresponding β chain sequence of HbA, and may represent a partial antigenic determinant specific for HbF.

In a small experimental series the radioimmunoassay was shown to be an accurate technique for measuring % of HbF in peripheral blood in the clinical laboratory. It was shown to be more sensitive than the alkali denaturation method at low levels of HbF and does not suffer from the disadvantage of the differential reactivity of hemoglobin F in different oxidation states in that method.

The total amounts of hemoglobin and the %HbF has been measured in two series of bursts grown in cell cultures of erythroid progenitors from human umbilical cord blood. Bursts contained approximately 10^{-12} moles of hemoglobin dimer. The %F content did not follow a normal distribution but was skewed toward higher values of HbF content with the possible existance of other modes as well. In preliminary experiments bursts grown from adult peripheral blood precursors contained an average of 3×10^{-13} moles of hemoglobin dimer per burst and much lower levels of HbF than of HbA.

Significance to Biomedical Research: The δ chain of HbF may have arisen by gene duplication from the β chain of HbA. No differences which might account for the selective advantage of HbF over HbA have been found. Studies of the conformation and surface structural characteristics of HbF may shed light on these differences. This can be approached by using specific antibodies to HbF and through crystal structure studies. In addition, antibodies specific to HbF may be used to quantitate small amounts of HbF and, thus, replace less quantitative methods now in use. These antibodies should be useful in prenatal diagnosis of abnormalities of hemoglobin production and sickle cell disease, and in studies of the differentiation of erythroid cells.

Proposed Course: Further experiments will be done to determine the

appropriateness of these immunological methods for quantitating the hemoglobin species present in samples obtained for antenatal diagnosis of sickle cell disease and β-thalassemia. Specific radioimmunoassays for HbF, HbA and HbS will be used for this purpose.

We will also use the assay in immunochemical studies of the nature of the HbF molecules as an antigen. To this end, other peptides representing specific sequences exposed on the surface of HbF in regions where there are differences from HbA will be synthesized.

The radioimmunoassays for HbA and HbF will be used further to follow the amounts of these hemoglobins in bursts of erythroid cells from different sources and under varying culture conditions.

No Publications

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,017-07 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immunochemical and Biochemical Studies of RNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. N. Schechter Chief, Section on Macromolecular Biology LCB NIAMDD

COOPERATING UNITS (if any)
Laboratory of Microbial Immunity, NIAID (Dr. Dan Eilat)
Arthritis and Rheumatism Branch, NIAMDD (Dr. Alfred Steinberg)
Department of Biochemistry, Washington U., St. Louis (Dr. K. Thomas)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,020-04 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Radioimmunoassay of Creatine Kinase: A Model for Radioimmunoassay of Human Serum Enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A.N. Schechter Chief, Section on Macromolecular Biology LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 AM 25,021-04 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Sickle cell anemia: Deoxyhemoglobin S Solubility and Cell Sickling Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.T. Noguchi	Staff Fellow	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

Laboratory of Biochemistry, NIDR (Dr. D. Torchia)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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0.6	0.6	

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SUMMARY OF WORK (200 words or less - underline keywords)

The gelation of deoxyhemoglobin S is believed to cause the sickling of SS erythrocytes. The solubility assay for characterizing a deoxyhemoglobin S gel was used to search for potential antisickling agents. We found that although several peptides from the hemoglobin sequence enhanced gelation, the β^{82-91} peptide could inhibit gelation of deoxyhemoglobin S to the same extent as phenylalanine and the β^{84-85} (Thr-Phe) peptide.

To study intracellular gelation, we developed an assay using ^{13}C double-resonance techniques. Data on samples of deoxyhemoglobin S gels obtained using ^{13}C NMR were correlated with data obtained using the sedimentation technique on the same samples. A correction to the deoxyhemoglobin S solubility obtained from sedimentation was necessary due to concentration gradients introduced during ultracentrifugation. Having thus found agreement between the two techniques, studies of intracellular-gelation as a function of oxygen pressure on SS erythrocytes was begun.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: A therapeutic antisickling agent for sickle cell anemia should retard gelation sufficiently to prevent sickling of an SS erythrocyte traveling through the arterial and venous circulation and back to the lungs to become reoxygenated. Studying the interaction of hemoglobin S with small peptides, particularly those from the hemoglobin sequence, should give us information about their ability to inhibit gelation as well as information about intermolecular interactions.

The solubility-kinetic assay for deoxyhemoglobin S is a precise, reproducible method for screening a variety of potential antisickling agents. However, it cannot be used to monitor gelation within the SS erythrocyte. Natural abundance ^{13}C NMR offers a way of characterizing gelation of deoxyhemoglobin S in solution as well as in the SS erythrocyte. Using scalar-decoupling, dipolar-decoupling and proton-enhanced decoupling methods, the amount of free hemoglobin and polymerized hemoglobin can be determined. Because the sample does not have to be separated into a supernatant and pellet phase, the data does not need to be corrected for artifacts introduced during ultracentrifugation.

Methods Employed: Large amounts of hemoglobin S have been prepared by ion exchange chromatography from units of SS, SC and SA blood. The method of optically monitoring turbidity and ultracentrifugation developed by Hofrichter, Ross and Eaton has been adopted. In this assay, concentrated hemoglobin S (close to the concentration found in red blood cells) is deoxygenated with sodium dithionite in a tube sealed under nitrogen. The kinetics of gelation are monitored by observing turbidity changes at 800 nm at 30°C - a delay period in gelation is observed and measured. The sample is then spun at 30°C in a thermally controlled ultracentrifuge at high speed which collapses the gel into a pellet leaving a solution saturated with tetrameric hemoglobin. The hemoglobin saturation at that temperature is obtained from measurements on a Cary 17 of the supernatant concentration. Fragments from several sites of the beta hemoglobin chain believed to participate in gel formation as suggested by recent X-ray crystallography data have been previously synthesized by a modification of the Merrifield method.

For ^{13}C NMR, samples are prepared in 8 mm NMR tubes, deoxygenated with sodium dithionite (buffer without sodium dithionite is used in the control sample) and sealed with a 5 mm tube cap via a ground glass joint. The 5 mm tube allows spectrophotometric determination of the extent of deoxygenation after the NMR spectra are accumulated. Natural abundance ^{13}C NMR with scalar-decoupling, dipolar-decoupling and cross-polarization yields a direct measure independent of spectral assignments and can be used on hemoglobin S lysate, chromatographed hemoglobin S and on whole cells. For the deoxyhemoglobin S solutions, after the ^{13}C NMR data were collected, the hemoglobin S samples were anaerobically transfered into quartz tubes for the sedimentation assay. The extent of deoxygenation was checked before and after transfer and deoxygenation was maintained. The supernatant concentration after sedimentation was determined.

Major Results: Many oligopeptides from the amino terminal region of the hemoglobin S β-chain enhanced gelation, probably as a result of excluded volume effects or binding of free water. However, the β^{82-91} peptide inhibited gelation, which we predicted on the basis of X-ray crystallographic data indicating an intermolecular contact in the region of β^{85} Phe.

^{13}C NMR has been used to characterize the deoxyhemoglobin S gel. The polymer fraction for deoxyhemoglobin S ranging in concentration from 21 g/dl to 32 g/dl was determined. The polymer fraction was also determined by ultracentrifuge sedimentation on the same samples and using the two phase model. The latter measurements were on the average .15 more. The discrepancy between the two techniques is largely removed by taking into account a correction on the order of 5% due to depletion of free hemoglobin in the sol phase as a result of ultracentrifugation. The corrected sedimentation data and data from the proton-enhanced spectra indicate a deoxyhemoglobin S solubility of 17.3 g/dl at 30°C. The correlation of data obtained using both techniques helps to establish ^{13}C double-magnetic resonance techniques as useful probes in studying gelation of deoxyhemoglobin S.

Significance to Biomedical Research: Peptide-protein interactions can be used as a probe to study the surface structure of normal and mutant hemoglobins. Of particular interest is hemoglobin S which aggregates to form a viscous gel under deoxygenated conditions. Increasing the delay time of gelation and the solubility of hemoglobin S are the minimal criteria for a suitable drug in sickle cell anemia therapy. In addition to phenylalanine and Thr-Phe (β^{84-85}), the β^{82-91} peptide of hemoglobin also inhibits gelation. The inhibition may possibly be due to the participation of the β^{85} region in an intermolecular contact in the deoxyhemoglobin S gel as suggested by X-ray crystallogrpahy. The enhancement of gelation by the β^{s} peptides indicate that the interactions of additives with water and solvent can also compete with the hemoglobin interactions to alter the gelation, and both solvent and additive effects must be examined more closely.

^{13}C NMR is an alternative technique for characterizing a deoxyhemoglobin S gel. The technique can also be used to study intracellular gelation as the samples are not centrifuged at high speeds as in sedimentation. The ability to monitor gelation within the SS erythrocyte enables one to examine intracellular gelation under a variety of conditions such as at different pH and oxygen tension values, and in the presence of inhibitors of gelation.

Proposed Course: The search for antisickling agents will continue considering aromatic amino acid-like compounds and other peptides. Interactions between hemoglobin and peptides will be examined more closely. Specifically, X-ray crystallography will be used to determine the binding site of phenylalanine and other small peptides. Radioactively labeled phenylalanine will be used to determine how much phenylalanine is incorporated into the polymer and to determine the binding constant between phenylalanine and hemoglobin S.

^{13}C NMR will be used to probe solutions of deoxyhemoglobin S and ^{13}CO-hemoglobin S, to determine the participation of CO-hemoglobin S in gelation.

The sedimented pellet phase will also be examined to determine the partition between free and polymerized hemoglobin S in the pellet. ^{31}P NMR will be used to study DPG binding to hemoglobin S and its participation in the gel. The ^{13}C NMR project will continue to search for optimum conditions to study intracellular gelation with regard to changes in pH, cation and anion flux, DPG concentration, and calcium concentration. Refinements of these techniques will be necessary in order to study the kinetics of sickling. Using ^{13}C NMR intracellular gelation as a function of oxygen tension and in the presence of inhibitors of gelation will also be studied. Samples of erythrocytes from individuals varying in severity of sickle cell anemia will also be studied using ^{13}C NMR to correlate intracellular gelation with the severity of the disease. ^{13}C NMR and kinetic-solubility assay for deoxyhemoglobin S remain useful tools in assessing the antisickling potential of a number additives. The kinetic-solubility assay is important for wide screening and study of hemoglobin S aggregation inhibitors. ^{13}C NMR offers an alternative method for also studying gelation and permits the study of SS erythrocytes and gelation in situ.

Publications:

Noguchi, C.T. and Schechter, A.N.: Inhibition of Sickle Hemoglobin Gelation by Amino Acids and Their Analogs. Biochemistry 17: 5455-5459, 1978.

Schechter, A.N. and Noguchi, C.T.: Inhibition of Sickle Hemoglobin Gelation by Peptides. In Beuzard, Y., Hercules, J. and Rosa, J. (Eds.) Development of Therapeutic Agents for Sickle Cell Disease. INSERM Symposium 9, Elsevier-North Holland, 1979, pp. 129-138.

Sutherland, J.W.H., Egan, W., Schechter, A.N., and Torchia, D.A.: Carbon-13-Hydrogen-1 Nuclear Magnetic Double-Resonance Study of Deoxyhemoglobin S Gelation. Biochemistry 18: 1797-1803, 1979.

Noguchi, C.T., Torchia, D.A., and Schechter, A.N.: ^{13}C nuclear magnetic resonance quantitation of polymer in deoxyhemoglobin S gels. Proc. Natl. Acad. Sci. U.S., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,022-03 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Specific Interaction of Human C3 with Erythrocytes and Leukocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.F. Tack	Guest Worker	LCB NIAMDD
	P.E. Lorenz	Guest Worker	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Interaction of complement protein C3 with specific receptors present on leukocyte and lymphoid cells is important to the modulation of immune response and immune surveillance pathways. Our interests in these processes have been two-fold: 1) to measure the kinetic and equilibrium parameters of interaction between C3 and its several activation fragments with their receptors on human cells and 2) to identify the chemical mechanism by which C3 attaches to a target cell membrane. The activation fragments C3b, C3c, and C3d have been purified from limit proteolytic digests of human C3. C3 and C3d have been radiolabeled and specific binding to human erythrocytes and B lymphocytes measured by direct methods. The binding of either ligand was a saturable reaction with an apparent association constant of 1×10^7 M^{-1}. Specific antibodies have been prepared to each of these fragments and used to develop sensitive radioimmunoassays. We are presently investigating the possibility that a covalent bond between C3b and the membrane may be formed by transesterification. We have detected a single sulfhydryl group upon either enzymatic conversion of C3 to C3b or treatment of C3 with hydroxylamine or hydrazine. We hypothesize that this sulfhydryl group is important to the activation of an acyl group within the C3 protein.

Project Description:

Introduction: Surface receptors specific for activation fragments of C3 have been detected on blood and tissue cells of many vertebrate species by a rosette assay. Two distinct receptors have been identified and shown to bind to topographically distinct regions of the C3 molecule: these include 1) the C3b receptor (CR1) present on the polymorphonuclear leukocyte, macrophage, B lymphocyte, and primate erythrocyte and 2) the C3d receptor (CR2) present on the macrophage and B lymphocyte. The primary function of CR1 and CR2 on immune surveillance cells is to facilitate contact with particles targeted for internalization. Recent studies of immune response pathways have shown an obligatory C3 requirement for complement receptor positive B lymphocytes to respond in vivo and in vitro to T cell dependent antigens suggesting that CR1 and CR2 may be required for T cell-B cell cooperative responses, perhaps at the macrophage level.

C3 deposited on a target cell membrane surface as a consequence of alternative or classical pathway complement activation remains associated with surface components on exposure to high salt, extremes of pH and temperature, reducing agents, and general protein denaturants. This binding has therefore been considered irreversible and probably covalent in nature. Recent studies have shown that nucleophilic reagents such as hydroxylamine and ammonia effect the dissociation of C3b from membrane surfaces and has prompted the speculation that attachment of C3b involves the formation of an ester bond.

Objectives: We are presently studying in a quantitative manner the binding of C3 and its activation fragments to their respective receptors on human leukocytes and primate erythrocytes. These studies have involved 1) the generation and subsequent purification of C3b, C3c, and C3d from limit proteolytic digests of the precursor protein, 2) the development of a direct binding assay for measuring the kinetic and equilibrium parameters of the binding of these fragments to cells, and 3) the isolation of antibodies to defined regions of the C3 molecule to be used as probes for the localization of receptor binding sites in C3b by specific antibody inhibition experiments and for the development of radioimmunoassays for each of the major activation peptides. We are also attempting to identify the principal amino acid residues important to our hypothesis concerning "acyl group activation and transfer" as a specific mechanism for attachment of C3b to membrane surfaces.

Results: The activation fragments C3a (9,200 daltons), C3b (179,000 daltons) C3c (147,000 daltons) and C3d (32,000 daltons) have been prepared by sequential digestion of the native protein with bovine pancreatic trypsin and porcine pancreatic elastase. These enzymes were chosen on the basis of preliminary experiments which indicated that the resultant activation products were indeed similar in their molecular weights and immunoelectrophoretic mobilities to the "physiological" C3 fragments which have been isolated, albeit in poor yield, from activated sera.

C3 and C3b have been radiolabeled with Na ^{125}I and the interaction with erythrocytes and lymphocytes measured using a direct binding assay. Equilibrium binding studies have been performed at 24°C in isotonic Tris:HCl buffer (pH 7.4) in the presence of bovine serum albumin at 10 mgm/ml. Preliminary kinetic experiments indicated that equilibrium conditions were attained at 1 hr. and that there was no significant change in the amount of bound ligand/cell up to 3 hr. of incubation. The separation of bound and free ligand was obtained by layering the cell suspension on a column of synthetic oil (ρ = 1.033 gm/cc) and pelleting the cells by spinning in a rapidly acclerating centrifuge. The nonspecific cell associated component was evaluated using as a control cells to which a 40-fold molar excess of unlabeled ligand was added. Initial studies have indicated that the binding of C3 and C3b to human red blood cells is a saturable reaction between 0.2 and 8.4 x 10^{-7}M ligand with an association constant (K_a) of 1-2 x 10^7 M^{-1}. The number of receptors/cell was estimated to be 1x10^3. C3b binding to an established human lymphoblastoid cell line has also been shown to be saturable with a K_a of 0.7-1.0 x 10^7 M^{-1}. The number of receptors/cell was estimated to be 1.5 - 2.0 x 10^4.

Antibodies to C3 and each of the activation fragments have been successfully raised in both sheep and rabbits. We have developed a precise radioimmunoassay for C3 using sheep antisera and a burro-antisheep antibody to separate free from bound. This assay is sensitive in the 10^{-10} to 10^{-9} M range. We are now developing radioimmunoassays for each of the fragments. In addition, these fragments are being coupled to Sepharose 4B and the resultant conjugate used as a specific immunoadsorbent with which to obtain specific antibodies from antiserum made to the native protein. The development of radioimmunoassays for C3 and its several fragments may have clinical application as well as importance in in vitro studies of the complement pathways.

We have observed that the same nitrogen nucleophiles (i.e. hydroxylamine and ammonia) effective in dissociating C3b from cell surfaces also abrogate the ability of C3 to bind to target cell surfaces. Concomitant with the loss of this binding ability the appearance of a single sulfhydryl group can be followed by titration with p-chloromercuribenzoate, alkylation with 1-^{14}C-iodoacetiamide, and disulfide interchange with 5,5'-dithiobis (2-nitrobenzoic acid). Electrophoresis of the radiolabeled protein on a SDS:polyacrylamide slab gel under reducing conditions has shown that the sulfhydryl group is uniquely associated with one of the two polypeptide chains which comprise this molecule. Further, limited proteolytic digestion of the labeled protein has localized the sulfhydryl group to the C3d fragment. Acid hydrolysis of the radiolabeled protein followed by high voltage paper electrophoresis has conclusively established the labeled amino acid to be S-carboxymethylcysteine. These sulfhydryl reagents have also been used to follow the appearance of this cysteine residue on proteolytic activation of C3 to C3b. Therefore, we suspect that a cysteine residue is important to the activation of an acyl group within the C3 protein, perhaps through formation of an internal thioester bond, and its transfer to unknown nucleophilic components on the membrane surface. Experiments are in progress to identify the acyl group donor to this putative thioester bond.

We have also improved methods for isolating early (C2, C3, C4) and late (C5, C6, C7, C8 and C9) complement components from human plasma and have isolated large amounts of most of these at high purity.

Significance to Biomedical Research: The complement proteins are significantly involved in host resistance to infection, in lymphocyte function and in autoimmune diseases. The C3 protein is central in the complement activation paths. Our studies of the structural chemistry of C3 activation, the topography and mechanism of non-covalent binding of C3 to cell surfaces and the chemistry of the covalent binding of C3b to cell membranes are all part of a broad program to use the methods of protein chemistry to probe the molecular basis of physiolgical and pathological processes involving this protein.

Presently, several papers are in the final stages of preparation and include: 1) a structural and immunochemical characterization of C3 activation, 2) the identification of a cysteine residue participating in acyl group activation and transfer, 3) quantitative aspects of C3 binding to receptors present on human red blood cells and a human lymphoblastoid cell line, and 4) improvements in the purification of the complement proteins.

No Publications

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,023-03 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Cell-cell Interactions in Protozoa. Isolation and Characterization of Mating-type Substances (M-agglutins)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edward Steers, Jr.	Research Biologist	LCB NIAMDD
	Audrey Barnett	Guest Worker	LCB NIAMDD
	Clifford E. Lee	Physical Science Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.8	0.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Conjugation (or mating) in Paramecium (as in numerous other species of protozoa and bacteria) occurs between complementary cells during special periods of reactivity. The interaction between reactive cells of opposite mating type shows an unusually high degree of specificity. When cells of opposite (complementary) mating type are mixed in culture, they adhere to one another on contact forming clumps containing as many as twenty cells. Within minutes of contact, the initial agglutination reaction is followed by pairing of complementary cells which separate from the initial clump. The complementary pairs undergo a fusion of their membranes along the oral surface which is followed by a reciprocal exchange of gametic nuclei and eventually mitosis. The process of specific cell agglutination and the subsequent events leading to mitosis are reminiscent of the biological actions associated with another class of agglutinus - the lectins. However, it now appears that the mating-type substances in paramecia are non-conjugated proteins. The project outlined in this report is designed to isolate and chemically characterize the substance(s) involved in the highly specific cell recognition and agglutination reaction referred to as mating.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: tinins) from P. multimicronucleatum. To characterize at the molecular level the chemical basis for the specific cell-cell interaction and agglutination between complementary M-agglutinins.

Methods Employed: Culture methods have been adapted to grow large volume (10-100 liter) quantities of mature cells in both reactive and unreactive states as well as smaller volumes (110 liter) of immature, unreactive cells. A procedure has been developed for the isolation of various subcellular fractions including purified reactive cilia from mature cells. A wide range of antisera has been developed against a variety of cells, subcellular fractions, and protein extracts against both unreactive and reactive cells. Immunologic analyses have been developed employing specific absorption tests, immunodiffusion and immunoelectrophoresis for studying the particular cellular fractions under investigation.

Procedures to isolate and purify the M-agglutinins from cellular extracts include a variety of methods including ammonium sulfate precipitation, gel filtration and ion-exchange chromatography and isoelectric focusing in sucrose gradients. Immunoaffinity chromatography has been developed as an alternate procedure for purification of the M-agglutinins. Biological assays have been developed using isolated reactive cilia to determine mating reactivity and localization of reactive sites. Immunological assays have been developed using specific immune sera. The inhibition, as well as induction, of agglutination of reactive cells is carried out in special depression slides and monitored microscopically.

Major Findings: When cells of complementary mating type are mixed they undergo a specific agglutination as a first step in a series of events which ultimately result in a reciprocal exchange of nuclei followed by mitosis. The cell-cell interaction is highly specific and involves only the cilia localized along the oral surface of the cells. Following agglutination, the cells separate from the clumps forming pairs. Pair formation is characterized by a fusion of the membranes along the oral suture. The initial recognition by comple-mentary cells through ciliary contact has lead us to develope a procedure for the isolation of reactive cilia free from cell bodies or other cellular debris.

In addition, a series of antisera have been developed against various types of cells (immature, mature-non-reactive, and mature-reactive) and cell-free fractions of reactive cells. Using certain antisera developed against reactive cells of mating type III, immunoaffinity columns were prepared by coupling purified IgG to activated Sepharose 4B. The buffer-soluble extracts from reactive cells (designated DBE) were passed through immunoaffinity columns and the retained material was eluted with glycine-NaON buffer, pH 10.2. The bound material represented a 5-fold increase in purification over the starting material. Further steps in the purification of affinity-bound material are currently underway which include isoelectric focusing. The buffer extracts from reactive cells have also been fractionated on DEAE cellulose into six major fractions. Immunological assays of

these fractions indicate the presence of mating-antigen in two separate fractions. Subsequent absorption tests using several different antisera which block mating suggest that two different mating antigens may exist in whole-cell buffer extracts.

In parallel studies designed to inhibit the specific cell-cell interaction, a survey was undertaken using proteases, glycosidases, lectins and sugars that are known to inhibit such interactions. We confirmed that the proteolytic enzymes trypsin and chymotrypsin specifically inhibit the mating reaction while four different glycosidases: dextranase, neuraminidase, ß-galactosidase and a mixed glycosidase from Diplococcus pneumoniae were ineffective. In addition, the series of lectins and sugars also failed to inhibit the mating reaction in anyway.

Significance to Biomedical Research: Specific cell-cell interaction is a universal phenomenon which is important in understanding various aspects of cellular development and behavior. Mating in Paramecium is characterized by a highly specific interaction between cells of opposite mating type leading to an agglutination reaction and cell fusion. Knowledge of the molecular basis for such cell specificity is important in understanding a variety of cellular interactions as well as gaining an understanding of the architecture of cell surfaces associated with such reactions.

Proposed Course: Having identified certain cilia as the primary [illegible] in the initial recognition and agglutination reaction of complementary cells together with two antigens located on the cortical membrane as potentially involved in pairing and membrane fusion, experiments will continue designed to purify the specific substances and localize them within the membrane. In addition, a survey of various potential inhibitors of cell-cell interactions will continue in an effort to gain an insight into the nature of the reaction at the molecular level. Ultimately, we hope to fully characterize the specific agglutination substances by various physico-chemical techniques and identify the nature of the reactive groups. Subsequent studies will include efforts to follow the synthesis of mating substances relative to the cell cycle and those factors which induce synthesis and trigger mitosis resulting from cell-cell interactions.

No Publications

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,025-03 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

A Study of the Mechanism of Folding of Cytochrome c Using Complementary Fragments

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.R. Parr	Senior Staff Fellow	LCB NIAMDD
	H. Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Protein Conformation Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Biologically functional, non-covalently linked complexes of cytochrome c are generated from several combinations of overlapping and non-overlapping fragments. The use of fast kinetic techniques to study the dynamic events leading to the formation of an ordered structure suggested that the process occurs in at least two distinct phases. We observed an initial, second order reaction in which the fragments combined to form a product having a structure reasonably similar to native cytochrome c. Additional evidence indicated that achievement of a native-like ligation state of the heme iron was a distinct kinetic event occurring after the second order reaction. This proposition has been tested by using chemically modified derivatives of the original fragments. These modifications prevent formation of a ligation state identical to that of native cytochrome c. In one instance the iron atom is removed from the heme moiety and in the other the methionine residues are converted to methionine sulfoxide. These derivatives exhibit second order kinetic behavior nearly identical to that of the original fragments. This provides strong support for the idea that formation of the native-like structure occurs in at least two kinetically distinct phases.

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ALL PAGES MUST BE NUMBERED IN SEQUENTIAL ORDER.

Project Description:

Objective: To elucidate the dynamic events involved in protein folding using the cytochrome c fragment complementing system.

Methods Employed and Major Findings: Three principal heme containing fragments consisting of residues (1-25)H, (1-38)H, and (1-53,55)H and four principal apofragments consisting of residues (54,56-104), (39-104), (23-104) and (1-104) have been prepared from horse heart cytochrome c. It is found that any given heme fragment will react with any overlapping apofragment and with its non-overlapping complement to form a non-covalently linked, yet biologically functional molecule resembling closely native cytochrome c. In addition, heme fragment (1-38)H will form a productive complex with apofragment (56-104) despite the fact that 17 amino acid residues are missing. On the other hand, combination of fragments (1-25)H and (39-104) results in much weaker binding and no biological activity.

Characterization of the productive complexes by spectral properties and by biological activities reveals only minor differences between them. This suggests that the end product is essentially the same despite the variability of the starting materials. This situation offers an outstanding opportunity for investigation of the dynamic events leading to the formation of tertiary structure in globular proteins.

Investigation using stopped-flow techniques has indicated that the formation of these complexes occurs in at least two kinetic phases as evidenced by differences in the kinetic profile observed by fluorescence and absorbance, and has suggested that ligation of methionine 80 occurs in the second phase of the reaction. In addition, a major difference in the rates of complex formation exhibited by (1-25)H and (1-38)H was attributed to intramolecular ligation of a second histidine residue in the case of (1-38)H. Addition of imidazole to the reaction mixture was thought to disrupt this ligation, resulting in very similar reaction rates for both heme fragments. These ideas have now been further tested by using chemically modified derivatives of the complementing fragments. These modifications prevent formation of a ligation state identical to that of native cytochrome c.

Porphyrin Fragments. When heme fragments are treated with hydrofluoric acid, the iron atom is removed from the heme moiety resulting in porphyrin fragments which are otherwise identical to the starting material. This modification clearly abolishes any ligation capability. Two such fragments have been prepared, (1-25)P and (1-38)P. Since the heme iron (via its ligands, His 18 and Met 80) is a significant factor in stabilizing the structure of native cytochrome c, complexes formed from porphyrin fragments would not be expected to have the same stability as productive complexes formed from heme fragments. However, it was anticipated that porphyrin complexes might have a stability similar to that of a non-productive heme fragment complex, (1-25)H:(39-104). This was indeed found to be the case, and it was concluded that a study of the kinetic parameters of porphyrin complex formation could provide insights into the role of the iron atom in the kinetics of heme fragment complex formation. The second order rate

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TYPING GUIDE SHEET

constants for formation of porphyrin complexes were found to be virtually identical for both (1-25)P and (1-38)P. Furthermore, the addition of imidazole to the reaction mixture had no detectable effect on the rate constants. These results provide strong support for our previous interpretation of the observed rate differences for (1-25)H and (1-38)H and of the effect of added imidazole. We also found that the second order rate constant for formation of porphyrin complexes was nearly identical to that for formation of a non-productive heme fragment complex, (1-25)H:(39-104). Since this rate constant is similar to that for productive (1-25)H complexes, these results provide strong support for the idea that the ligation state of the iron atom does not change during the second order phase of heme fragment complex formation. However, since the ligation state of the final product is indeed different from that of the starting heme fragment in productive complex formation, at least two kinetic phases are implicated in the overall reaction mechanism.

Methionine Sulfoxide Apoprotein. When apoprotein (1-104) is treated with chloramine-T under the proper conditions the methionine residues are quantitatively converted to methionine sulfoxide, while the rest of the polypeptide chain remains unaltered. This modification effectively prevents ligation of the sulfur atom of methionine 80 to the heme iron atom, an important structural feature of native cytochrome c. Nonetheless, methionine sulfoxide apoprotein, (1-104MetSO) forms tightly bound complexes with heme fragments (1-25)H or (1-38)H. Based on circular dichroism spectra and fluorescence properties, the complexes appear to have an ordered structure very similar to that of native cytochrome c. Thus a kinetic study of the formation of [illegible] heme fragments and (1-104METSO) could provide additional insights into the mechanism of formation of productive complexes of cytochrome c fragments. When the kinetics of complementation of (1-104) with (1-25)H, (1-38)H, or (1-53)H are monitored via fluorescence quenching, a single, kinetically second order reaction is observed. However, when these same reactions are monitored by absorbance changes, the data are not adequately described by either first or second order kinetic equations. This led to the idea that at least two kinetic phases must be involved in the overall process, with the second phase producing additional absorbance changes but no further fluorescence changes. The fact that saturation of the heme iron with imidazole did not interfere with the second order reaction suggested that ligation of methionine 80 occurred in the second phase. When the kinetics of complementation of (1-104METSO) with (1-25)H, (1-38)H, or (1-53)H are monitored via fluorescence quenching, a single second order kinetic reaction is again observed, and the rate constants are very similar to those obtained with (1-104). However, when the reactions of (1-104METSO) are monitored by absorbance changes, the results are identical to those obtained by fluorescence quenching. Thus the blocking of methionine ligation has removed the complexity of absorbance kinetic profile. This result strongly supports our original interpretation of at least two kinetic phases in the formation of productive complexes, with ligation of methionine 80 occurring in the later phase(s).

Significance to Biomedical Research: The understanding of the interatomic interactions underlying the conformational dynamics and functions of proteins is important in the understanding of cellular mechanisms involving proteins. These fragment complementing systems of cytochrome c are an excellent test object for examination of the contribution of individual amino acid residues to the interatomic interactions responsible for the structure and function of cytochrome c, an important class of proteins.

Proposed Course of Research:

1) Kinetic experiments with the iron atom in the reduced (+2) state.
2) Study the temperature dependence of the rate constants.
3) A study of the factors affecting the relative ratio of (1-25)H:(23-104) to that of (1-38)H:(56-104) formed on mixing (1-38)H and (1-104).

Publications:

Parr, G.R., Hantgan, R.R. and Taniuchi, H.: Formation of Two Alternative Complementing Structures from a Cytochrome c Heme Fragment (Residues 1-38) and the Apoprotein. J. Biol. Chem. 253, 5381-5388, 1978. (Reported as in press in Z01 AM 25,025-02 LCB.)

Parr, G.R., and Taniuchi, H.: A Kinetic Study of the Formation of Ordered Complexes of Ferric Cytochrome c Fragments. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,026-02 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Location of the Antigenic Determinants on Normal and Sickle Hemoglobin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Alston	Staff Fellow	LCB, NIAMDD
	A. Eastlake-Dean	Chemist	LCB, NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.1	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are using quantitative immunochemical methods to locate the antigenic determinants on hemoglobins A and S. We have changed the metal in the porphyrin of the heme group in both hemoglobins and are studying these reconstituted hemoglobins by quantitative precipitin and radioimmunoassay to see if any significant conformational changes have been induced. These studies will allow us to detect subtle changes in the conformation of hemoglobins with zinc, copper, cobalt, manganese and nickel metalloporphyrins. Further, the metalloporphyrin and the chemical modification studies should allow us to locate antigenic determimants on the hemoglobin molecule. In addition we are studying the effects on the gelation of sickle hemoglobin of the metalloporphyrin substitutes. A solubility assay in buffers of high phosphate concentration is being used for this purpose.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The gelation of hemoglobin S has been of continuing interest to this and other laboratories. Locating the antigenic sites on the hemoglobin molecule may facilitate the design of a specific compound that can be synthesized, or of some complementary peptide fragment that can be isolated, which will interact with the hemoglobin surface and inhibit gelation. To study these antigenic determinants, we are measuring the binding of antibodies to hemoglobins A and S by quantitative precipitin and radioimmunoassay methods and studying the effects of chemical modifications and peptide fragments on the antibody-hemoglobin interactions.

In addition we have been preparing a variety of metalloporphyrins, with other metals substituted for the iron atom and are reconstituting hemoglobins A and S with these prosthetic groups. We are using the immunochemical methods to study the effects of these substitutions on the hemoglobin structure. We are also studying the ability of these metalloporphyrin hemoglobin S derivatives to gel.

Methods Employed: Hemoglobins A and S were purified from the blood of AA, SS, and SC donors by ion exchange chromatography. Normal hemoglobins A and S were reconstituted using copper, cobalt, zinc, manganese, nickel, protoporphyrin IX and the native iron. This was accomplished by removing the heme group yielding the apohemoglobin and reconstituting the protein with the above metalloporphyrin derivatives. The various metallohemoglobins were purified by column chromatography employing Sephadex and Carboxymethyl Cellulose. The hemoglobins were then concentrated, dialyzed against 0.05 M phosphate buffer, pH 7.1, frozen in liquid nitrogen and stored in a Revco freezer.

Antibodies against native hemoglobin A were obtained from sheep. The IgG component of the antisera was purified using ammonium sulfate and chromatographed on an anion exchange resin. The material was concentrated, frozen and stored as described above. The various hemoglobin derivatives were tested for their antigenic reactivity against the IgG using the standard Ouchterlony technique. Precipitin curve analyses were carried out on the metallohemoglobins to check further for conformational changes. A sensitive and average radioimmunoassay was used to analyze the conformations of these proteins.

In addition, a solubility assay using high concentrations of phosphate buffers has been established to test the tendency of these proteins to aggregate.

Major Results: The metallohemoglobin derivatives which contained copper (II), cobalt (II), manganese (III), apo and reconstituted iron (III) showed complete identity with antibodies raised against the native carboxyhemoglobin as shown by Ouchterlony studies. Protoporphyrin IX, nickel (II) and zinc (II) hemoglobin showed partial identity with the same antibodies. In quantitative precipitin curve analyses, copper (II), cobalt (II), and reconstituted iron (III) were observed to precipitate identical amounts of antibodies as the native protein. Manganese (III) and protoporphyrin IX

hemoglobins precipitated slightly less antibody than the native protein but the differences are not considered large enough to be significant. However apo, zinc (II) and nickel (II) hemoglobin precipitated significantly less antibody than the native protein in the order apo < nickel (II) < zinc (II). The results obtained from the radioimmunoassay showed that cobalt (II), manganese (III) and reconstituted iron (III) hemoglobin totally displaced all of the native hemoglobin from the antibody and showed the same affinity toward the antibody as native carboxyhemoglobin. Protoporphyrin IX and apohemoglobin showed less affinity for antibody than the native protein, however these derivatives still displaced all of the protein from the antibody. Copper (II), nickel (II) and zinc (II) showed the least affinity for antibodies to the native protein in the order zinc (II) < nickel (II) < copper (II). These derivatives also displaced all of the native protein from the antibody.

Significance to Biomedical Research: The various metallohemoglobin derivatives serve as unique probes in studying the active site of the native hemoglobin molecule and the concomitant effects transmitted to the rest of the protein moiety. These studies will help us understand what occurs when the iron atom in the heme group of native hemoglobin changes its electronic properties when undergoing the R T transition. The various metalloporphyrin derivatives allows us to separate the effects of changes in the ionic radius, electron spin, coordination sphere and spin density at the active site on the remainder of the protein molecule. We are studying these subtle changes by immunochemical techniques. Changes in the protein moiety could change some of the antigenic determinants and one can then begin a search for these determinants with hope that compounds which will interact specifically with one or more of the sites can be designed. In doing so the intermolecular contact points which exists when deoxhemoglobin S gels can be inhibited and this should decrease the amount of hemoglobin which polymerizes and therefore decrease the gelation which is responsible for the well known sickle crisis.

Proposed Course of Project: Since the radioimmunoassay copper (II), nickel (II), zinc (II) and protoporphyrin IX hemoglobins showed that some conformational change has taken place, we are now testing the solubility of these derivatives (A and S) in high ionic strength phosphate buffers to see if such changes cause an increase or decrease in their solubility as compared to the native proteins. We will then test to see if these compounds will have decreased gelation under in vitro conditions. The ultimate goal of the project is to locate the antigenic sites where these conformational changes have taken place and synthesize compounds (especially peptides) which are complementary to those sites which will have a high degree of specificity in inhibiting gelation.

No publications

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,027-02 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Cytochrome c Biosynthesis: Enzymatic Formation of Heme-apoprotein Covalent Bond

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Basile	Visiting Fellow	LCB NIAMDD
	H. Taniuchi	Chief, Section on Protein Conformation	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Conformation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to find a hypothetical enzyme (cytochrome c synthetase) which would catalyze the covalent bonding of heme to apocytochrome c, Saccharomyces cerevisiae is chosen as an enzyme source. Synthesis tests have been carried out using ^{125}I labeled apocytochrome c and heme as substrates, and the yeast mytochondria as presumably enzyme enriched cellular fractions. The extent of labelled apoprotein incorporation into the fraction of cytochrome c added as the carrier has been as much as 7% (0.35 nmol) of the extracted radioactive material. This radioactive material, resembling cytochrome c, will be further investigated in order to establish the exact nature of the observed phenomenom.

Project Description:

Objectives: Finding of an enzyme which catalyzes the formation of covalent bond between heme and apocytochrome c - hypothetical cytochrome c synthetase.

Methods Employed and Major Findings: ^{125}I labelled apocytochrome c was obtained from ^{125}I labelled yeast cytochrome c by the silver sulfate method (J.B.C., 248, 3188, 1973). Yeast cytochrome c (Sigma) was labelled as following. A mixture containing 2.8 mM cytochrome c, 2.5 mCi ^{125}I (Amersham), 5 µl of 5 mg/ml lactoperoxidase (74 u/mg), 8.8 mM H_2O_2 and 50mM potassium phosphate, pH 7.4 in a total volume of 490 µl was incubated at 25°C for 1 min. Then, the reaction was quenched by addition of 20 µl of ß-mercaptoethanol. The mixture was treated by gel filtration on a Sephadex G-50 column in 0.075M Tris/glycine, pH 8.9. The protein fraction thus obtained was dialyzed against H_2O.

On the basis of the available information and the location of the enzyme involved in the last step of heme biosynthesis and that of cytochrome c, mitochondria are used as the most likely source of the enzyme. Starting from yeasts grown in 10 liters of the growth media under aerobic conditions as described in Biochem. Biophys. Acta 95, (1965) 486, yeast cells were harvested and lysed by a hypotonic solution after cell wall having been digested with ß-glucuronidase-arylsulfatase (J. Bacteriol. 88, (1964) 1762). Mitochondria were then isolated according to the published method (J. Bacteriol. 88, (1964) 1762.

For the experiments of biosynthesis of cytochrome c, mitochondria were incubated in the presence of heme and the ^{125}I labeled apoprotein under varying conditions in pH, O_2 pressure, presence of cofactors such as NADH, ATP etc. The effect on the incorporation of ^{125}I labelled apoprotein into the cytochrome c of both sonnication and French press treatment to disrupt mitochondria, has also been tested. After a suitable incubation time, carrier cytochrome c was added to the incubation mixture, and the radioactivity associated with the cytochrome c, was measured. In the best case 7% apoprotein incorporation into cytochrome c has been observed after the following purification steps:

1) Co-elution with native cytochrome c of the radioactivity by gel filtration on a Sephadex G-50 column in the presence of guanidine HCl.

2) Subsequent adsorption of the radioactivity to an ion exchange column followed by gradient elution.

3) Trypsin digestion under reducing conditions of the eluted fraction of step 2) (ferrous cytochrome c is trypsin resistent).

4) Purification after step 3 by gel filtration on a G-50 Sephadex column (1x210 cm).

Disruption of mytochondria with both the methods described above decreased the yield of ^{125}I-labelled cytochrome c-like product. An improved method for purification of cytochrome C will be adopted in the future experiments. Its efficacy in terms of cytochrome c recovery and purity has already been tested. The increased recovery will permit further characterization of the radioactive cytochrome c-like product.

Significance to the Biomedical Research: The present study is of prime importance in clarifying the final step of biosynthesis of an important protein involved in cell respiration, as much as in photosynthetic metabolism. It is therefore pointed out that the information of the present work with the system of yeast could be applied to the corresponding system of various organisms.

The availability of cytochrome c synthetase would open the way of the total chemical synthesis of cytochrome c for the study of structure-function of the protein.

Proposed course of research:

1) Identify the exact nature of the product obtained after incubation of ^{125}I labelled apoprotein and heme with the yeast mitochondrial fraction.

2) Increasing in the activity of yeast extracts in order to increase in incorporation of ^{125}I apoprotein into cytochrome c.

3) Purification and characterization of the cytochrome c synthesizing enzyme.

No Publications

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,028-01 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Sickle Cell Anemia: Design and Synthesis of Gelation Inhibitors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. Pavone	Visiting Fellow	LCB NIAMDD
	A. Eastlake-Dean	Chemist	LCB NIAMDD
	K.L. Luskey	Research Associate	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

Division of Computer Research and Technology, NIH (R.J. Feldmann)
Dept. of Chem., Cornell University, Ithaca, N.Y. (H.A. Scheraga)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Macromolecular Biology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	2.0	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The regions of contact between deoxyhemoglobin S tetramers in the crystal form have been analyzed with a computer graphics system. Peptides complementary to two of these contact regions have been projected and their interactions with the hemoglobin analyzed visually. Several of these peptides have been synthesized by solid-phase and solution methods and purified and characterized. These peptides have been tested on the kinetic and solubility assays of deoxyhemoglobin S gelation. All, so far, have a negative effect, probably due to excluded volume. New peptides are being designed and synthesized using energy minimization and other considerations to enhance their potential effectiveness as inhibitors of sickling.

To study in more detail the mechanism of excluded volume, we have tested a variety of short homopolymers of the most soluble amino acids. It has been found that lysine and serine peptides have the effect on activity coefficient predicted from their weight fraction; proline, however, has a lesser effect, suggesting a specific molecular interaction.

Project Description:

Objectives: Sickle hemoglobin under physiological conditions aggregates upon deoxygenation to form a viscous gel composed of long fibers. This form of aggregation within an SS erythrocyte is believed to result in the cell deformity and membrane rigidity characteristic of a sickled SS erythrocyte. Much effort has been devoted in the last years to identify the residues involved in the protein-protein interactions which might be responsible for the stability of the gels.

Since crystallization and formation of gels are both processes of aggregation a comparison has been attempted between the three dimensional structure of crystals of deoxyhemoglobin S and the results of electron microscopy and x-ray diffraction of fibers. Presently it seems reasonable to believe that the interaction sites among tetramers found in the crystals are quite similar in the gels. In searching for a therapeutic agent for sickle cell anemia we are looking for an additive which will specifically bind to the hemoglobin surface at the sites of interactions as found in the crystals and which could change the kinetic and/or the equilibrium between the isolated protein molecules and the gel.

Presently we think that peptides are the most suitable compounds. In fact small fragments of the native protein of the regions of interaction could lead to a specific binding for the complementary region of the protein surface, if those fragments will assume in solution the same conformation as the fragments in the native protein. The water solubility of these peptides may be altered by adding or changing some amino acids of the native sequence-hydrophobic or hydrophilic amino acids to decrease or increase the solubility, respectively. Peptides are naturally occurring compounds and might have low toxicity. Protection of the carboxyl terminal of the peptides with long hydrocarboxy-chains might increase membrane permeability. Furthermore, we have shown that phenylalanine-containing peptides and the isolated amino acid itself increase the deoxyhemoglobin S solubility, as well as do tryptophan and other hydrophobic compounds. We are using molecular structural information from the deoxyhemoglobin S crystal to design such potential inhibitors and are synthesizing those by solid-phase organic-chemical methods. The effectiveness of these peptides on the properties of the deoxyhemoglobin S gels is being tested.

Methods and Major Results: We have used the Display Graphics System of the NIH DCRT to study the molecular structure of the deoxyhemoglobin S crystal. The coordinates were suppled by Dr. W. Love of Johns Hopkins University based on 3 Å map of the protein. The surfaces of the hemoglobin S molecules at regions of intermolecular contacts in the crystal were examined and two of these regions, around β^{73} and β^{22}, were selected as being the most suitable for building complementary peptides.

The structures of about twenty possible complementary peptides for these two regions were projected on the computer graphics system and the interactions with the protein were examined by qualitative considerations of steric and charge properties. On this basis about ten peptides ranging

from three to nine residues, were chosen as likely candidates for competitors of the gelation reaction.

Five of these peptides Gly-L-Pro-NHB_2, Lys-Pro-His-Pro-Asp, Lys-Gly-His-Pro-Asp, Asp-Pro-His-Gly-Lys, Asp-Gly-His-Lys, were synthesized by us. One of these, Gly-L-Pro-NHB_2, was made by carbodiimide activation in solution. The other four peptides were synthesized using a modified Merrifield procedure with a jaketed reaction vessel in which nitrogen was bubbled for delicate mixing during each of the steps. A cloromethylated polystyrene bead resin was used as the solid phase and coupling of suitably protected amino acids by carbodimide activation was accomplished. The finished peptides were cleaved from the resin by HF treatment and deblocked further with piperdine, and benzenethiol. The products were purified by gel chromatography and ion exchange chromatography. The purity of the products, as established by TLC and by amino acid analyses, was found to be very high, as were the yields. In addition two peptides-Glu-Pro-His-Pro-Lys and Glu-Pro-His-Lys- were synthesized for us by Peninsula Laboratories, but were purified by us.

So far we have tested two of these finished products in the solubility and kinetic assays. (The solubility of three of the other five peptides has been a major difficulty so far.) Concentrated solutions of the peptides have been added to small tubes of deoxyhemoglobin S and the rate of gelation after a temperature jump and the final solubility as determined by near -IR spectroscopy after centrifugation has been ascertained. Unfortunately these two peptides decrease solubility, presumably due to an excluded volume ("salting-out") effect.

In order to better understand the behavior of peptides in a concentrated solution of hemoglobin S a series of experiments were carried out in which increasing amounts of amino acids and short homopolymers of amino acids were added to hemoglobin S and the kinetics of gelation and equilibrium solubility were measured. Lysine, dilysine, tetralysine, $lysine_{8-12}$, serine and hexaproline all behaved remarkably similar when the effect on hemoglobin S solubility is compared with relation to the weight amount of additive. This behavior is very similar to the effect observed by others of large globular proteins increasing the activity coefficient of hemoglobin S and thus decreasing its solubility. This behavior of these model compounds provide a background against which peptide additives must be compared to determined their effect on gelation. Thus, proline when compared to these other amino acids does not exhibit a great decrease in solubility of hemoglobin S and thus may interact with the protein and inhibit gelation, although to a minor degree.

In order to design peptides for the purpose of more specifically and strongly binding to the hemoglobin molecule, we have obtained the ELEEP Program for energy minimization from Dr. H. Scheraga and are using this to test more quantitatively the binding of our projected peptides with the hemoglobin molecules. In addition, we are now blocking the amino and carboxyl-terminal groups of the peptides to minimize the effects of these charges. These peptides will be tested soon in the gelation assay.

Significance to Biomedical Research: The intracellular gelation of sickle hemoglobin is the basis of the severe disease, sickle cell anemia.

Recent progress in understanding the molecular basis of gelation and the structure of the deoxyhemoglobin S gel and crystal has opened the possibility of designing specific inhibitors of gelation which might have ultimate therapeutic importance. We are studying peptides complimentary to the surface of the hemoglobin molecule for this purpose. The problem is a difficult one because of the very high hemoglobin concentration in the cell and the resulting non-ideality but it may be possible to find classes of peptides with significant inhibitory effect, greater than that of free phenylalanine for example. However, possible problems with cell permeability, toxicity, and biological life-time will also have to be dealt with. At present, this approach is among the most promising in this disease and serves as a general model for the inhibition of protein-protein interactions in other systems.

Proposed Course of Research: We plan to design, synthesize and test other groups of peptides as inhibitors for gelation with these more sophisticated computer analyses and peptide design considerations. New techniques to measure the binding of small molecules on the hemoglobin surface will be used as: differential spectroscopy, affinity chromatography, fluorimetry, differential scanning calorimetry and x-ray diffraction will be considered as possible means to demonstrate specific interactions.

Simgle crystal analyses by x-ray will also be used to test the close similarity between crystals and fibers and to understand better the relationship between hemoglobin crystallization and the sickling of sickle erythrocytes due to intracellular gelation and fiber formation. (These studies will use the eqipment of Dr. H. Taniuchi and Dr. D. Davies.) This research will be continued until it is clear whether or not this approach has therapeutic potential.

No Publications

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 25,029-01 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Molecular Mechanisms of Mammalian Fertilization

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Dean	Senior Investigator	LCB NIAMDD
Other:	S. Harwood	Bio-lab Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.6	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Immature mouse follicles are isolated and grown in culture for nine days and then biosynthetically labeled with radioactive amino acids or sugars. During this maturation period the zona pelluuda, an acellular matrix comprised of glycoproteins is formed around oocytes. The proteins of the oocyte and zona pellucida can be displayed on two dimensional O'Farrell gels and followed for changes during this period of oocyte development. Methods are being developed to isolate the zona pellucida from the oocyte and determine its protein composition. These proteins are thought to mediate the specie-specific sperm-egg interaction by acting as recognition markers and subsequent sperm binding sites. We hope to identify and characterize these proteins both as to their physical properties and site of synthesis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: We are interested in examining the molecular processes of mammalian fertilization and early development. As a first step in this longer term project we are undertaking to isolate and characterize the proteins in the murine zona pellucida that presumably mediate the specie specific sperm-egg interaction.

Methods and Major Results: At birth ova in the female mouse are in mieotic arrest and have no potential for mitotic cell division prior to fertilization. This leads to a paucity of biological material which can be partially overcome by radioactively labeling the protein of the maturing oocyte. To do this we have modified previously published methods for the short term in vitro culture of mouse follicles. Two to three thousand follicles are isolated from 8 day old DBA/2 females and grown in culture for nine days in such a manner that the granulosa cells spread in a monolayer and trap the oocytes underneath. During this period the eggs enlarge to a diameter of 65-70 μm and undergo a normal maturation process which includes the production of the zona pellucida. The zona is an acellular matrix, thought to be composed of glycoproteins, which surrounds the oocyte and has a diameter of approximately 5-7 μm.

Prior to harvesting, the eggs they are incubated for various times with radioactively labeled amino acids (^{35}S-methionine, ^{14}C leucine) or carbohydrates (^{3}H-glucosamine, ^{3}H fucose). The eggs are then lysed by freeze-thawing in the presence of 9M urea and NP-40. The lysed material is analyzed by two-dimensional electrophoresis using isoelectric focusing in the first dimension and SDS slab gels in the second dimension by the method of O'Farrell et al. Fluorography, by the method of Bonner and Laskey is used to detect radioactivity incorporation into proteins and to ascertain the variety of proteins as well as their changing patterns in the in vitro matured oocytes. Using spleen derived lymphocytes labeled in short-term culture with ^{35}S methionine we have been able to validate this methodology and can recognize more than 200 hundred different proteins. Methods are now being developed to physically isolate the zona pellucida from the egg that it surrounds so that a similar analysis of these glyco-proteins can be undertaken and the proteins that mediate sperm egg interactions identified.

Significance to Biomedical Research and Proposed Course of Project: An understanding of the molecular basis of mammalian fertilization would open the possibility of modulating the sperm-egg interaction. This in turn could have profound practical application in animal husbandry and, perhaps, in development of more effective means of prevention of world overpopulation.

In a biochemical sense this project represents a system in which cell-cell interaction and the proteins that mediate these specific interactions can be well studied. The oocyte proteins may act as receptors and bind sperm surface ligands in a reversible manner or they may irreversibly modify sperm surface proteins so as to facilitate binding and penetration

of the zona pellucida by the sperm. Subsequent fusion of the sperm with the oocyte represents an initial point in embryogenesis and cell division. Thus, murine fertilization is a model system of cell differentiation and early mammalian development. Studies on changes in surface and cytoplasmic proteins can be undertaken so as to begin to sort out the complex variables that regulate early mammalian differentiation.

We hope to use the in vitro culture of mouse oocytes not only to isolate and identify the function of the proteins in the zona pellucida but also to investigate the site and regulation of their synthesis. We also hope to be able to use the isolated zona proteins as probes to investigate sperm acrosomal proteins that are involved in fertilization. More long range projects will include examination of sperm penetration of the zona pellucida, sperm-egg plasma membrane fusion and the subsequent cortical reaction that presumably modifies zona proteins and prevents polyspermy.

No Publications

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,030-01 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Purification and *in vitro* Translation of Human Lymphoblastoid Messenger RNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: K. Zoon Staff Fellow LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology,

SECTION

Section on Protein Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Poly(A)-bearing RNA containing the interferon mRNA has been isolated from Namalva cells induced with Newcastle disease virus and translated in the Xenopus laevis oocyte system. The interferon mRNA has been purified 30-fold by sedimentation on a sucrose gradient under denaturing conditions with a 30% recovery and has a sedimentation value of 135 relative to globin mRNA. Using

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Interferon mRNA has been isolated from Namalwa cells induced with Newcastle disease virus. The cells are disrupted in hypotonic buffer containing the nonionic detergent Triton N101 in the presence of ribonucleotide-vanadyl complexes (S. Berger and C. Berkenmeier, submitted for publication) which minimizes degradation of translatable mRNA. The cytoplasm is extracted with phenol and then chloroform:isoamyl alcohol:phenol to obtain the RNA. The poly(A)-bearing RNA is isolated by chromatography using poly(U)-Sepharose and constitutes our mRNA. Using the Xenopus oocyte system 25-100 ng of mRNA yields 600-1000 reference units of interferon. No interferon was detected in the wheat germ or rabbit recticulocyte cell-free protein synthesizing systems when similar quantities of interferon mRNA were introduced. Studies are in progress to determine optimum conditions for maxmizing the yield of interferon mRNA from Namalwa cultures i.e. elapsed time between the induction and harvesting of the cells, cell density, and nutritional requirements at the time of induction. Fractionation of the interferon mRNA by sedimentation using a 70% formamide sucrose gradient results in a 30-fold purification of the interferon mRNA and a recovery of approximately 30%. The interferon mRNA has a sedimentation value of 13S using globin mRNA as a marker. Additional studies are in progress to further purify the interferon mRNA, prepare the cDNA, and subsequently clone the interferon genes.

Studies of incorporation of radioactive amino acids into interferon using the Xenopus oocyte system have been initiated. Oocytes are injected with either partially purified interferon mRNA or an analogous fraction of mRNA from uninduced cells and incubated with radioactive amino acids. The interferon from the oocytes will be purified by immunoprecipitation or a combination of steps previously described in the purification section. The radiolabeled interferon will be analyzed on a LKB sequenator. The amino acid sequence will be determined using HPLC to identify the positions of the radioactive amino acid phenylthiohydantoin derivatives in the polypeptide. By incorporating each of the 20 radioactive amino acids into the interferon, the sequence can be established.

Publication:

Berger, S., Hitchcock, M., Zoon, K., Friedman, R., Birkenmeier, C., and Chang, E.: Ribonucleoside-Vanadyl Complexes as Nuclease Inhibitors: Preparation of Interferon mRNA from Namalwa Cells. Proceedings of the International Workshop on Interferon, Sloan-Kettering, 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,031-01 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Preparation of High Specificity Antisera to Human Interferon and Then Use in Radioimmunoassays

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Smith	NIAID Fellow	LCB NIAMDD
	D. Zur Nedden	Bio-lab Technician	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Protein Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies of high specificity toward human lymphoblastoid interferon have been produced in sheep and rabbits. These have been used for preparation of high capacity immunoadsorbant affinity chromatography columns for use in purification of interferon.

Antibodies to interferon have also been produced by in vitro cultures of lymphocyte hybridomas. Experiments are now in progress to clone the hybrid cell lines in soft agar and produce monoclonal IgG or IgM for use in radioimmunoassay and receptor studies.

Using ^{3}H methylated interferon as labeled antigen, standard curves for quantitative immunoprecipitation assays have been made. With the present specific radioactivity of labeled interferon the radioimmunoassay can be sensitive to within 10-100 Units of interferon or 10-fold less than present bio-assay.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Antibodies to human lymphoblastoid interferon have been produced in sheep for the past three years. Recently, only highly purified interferon preparations have been used as immunogen in the sensitized sheep with resultant production of significantly higher titer of antibody and much greater specificity than was previously obtained. Sheep are subjected to plasmaphoresis and the plasma is clotted and delipidized with dextran sulphate. Dilutions of $1/10^6$ are capable of 50% neutralization of interferon activity. IgG has been purified from this antiserum and used to make an immunoadsorbent affinity column. This new column had a 10-fold increased capacity of interferon binding per ml of sepharose and an apparent association constant 3 to 4 magnitudes greater than those previously used in the purification of interferon. Some difficulty has been observed in recovering all of the interferon activity applied to the column, however, several different elution conditions are presently being evaluated to increase recovery and to take advantage of the increased affinity and specificity.

Antibodies have been produced in rabbits using as immunogen interferon of greater than 10^8 units/mg specific activity. This antisera has been used to prepare small scale immunoadsorbent affinity columns. The IgG from these antisera will be used for the immunoprecipitation in final steps of interferon purification.

Using purified sheep IgG anti-interferon and ^3H methylated interferon, immunoprecipitation assays and other preliminary work for the generation of a quantitative radioimmune assay for interferon has been done. ^{125}I labelled interferon has been found only very slightly reactive toward the sheep IgG. The use of ^3H-methylated interferon has been found sufficiently reactive toward the sheep IgG and with labelling procedures using NaB^3H_4 (specific activity 500 Curies/mmole) labelled interferon can be produced with a specific activity of 10^8 dpm/µg. With this high specific radioactivity, the interferon radioimmunoassay will be sensitive to within 1 to 2 orders of magnitude of the present bioassay for antiviral activity. The primary problem with the bioassay is the high standard error and this will be dramatically reduced with the use of a radioimmune assay.

BALB/c mice were immunized with highly purified interferon preparations and after three secondary boosts the spleen cells were hybridized with a sub line of the BALB/c mouse meyelome MOPC-21 (NS-1). The hybridization was performed by incubation with polyethylene glycol 1500 and the primary cultures were grown in microtiter trays and assayed for neutralizing activity toward interferon. Five out of 48 initial cultures were positive for anti-interferon production by neutralization assays. Screening procedures using modification of the method described by Sharon et al. (PNAS 76 1420, 1979) and the cloning procedures in soft agarose have been established. Currently, culture conditions have been developed for the maintainence both in vivo and in vitro of mouse meyeloma cell line Sp 2/0-Ag14, a cell line shown to be capable of efficient hybridization with production only of the spleen cell parent immunoglobulin and more of its own meyeloma proteins (Schulman et al., Nature, 276, 269, 1978). When viable clones are obtained which are positive for anti-interferon they will be grown both as in vitro cultures

and *in vivo* as peritoneal ascites tumors in BALB/c mice. The yield of IgG from one mouse tumor can be as much as 0.5-1 g of protein. This purified monoclonal immunoglobulin will then be used in interferon purification procedures, radioimmunoassays, and receptor identification investigations.

No Publications

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,032-01 LCB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Hemoglobin Synthesis in Cultured Erythroid Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.L. Luskey	Research Associate	LCB NIAMDD
	A.N. Schechter	Chief, Section on Macromolecular Biology	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Biology

SECTION
Section on Macromolecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fetal hemoglobin (or hemoglobin F) is the major hemoglobin produced during the later part of fetal life. The genes encoding for the gamma chain (the non-alpha chain of hemoglobin F) and the beta chain (the non-gamma chain of hemoglobin A) are in close proximity on chromosome 11. At birth, hemoglobin F is replaced by hemoglobin A as the predominant protein within the red cell. However, throughout life small levels of hemoglobin F persist in a small population of cells designated as F cells.

We are studying the expression of hemoglobin synthesis in cultures of erythroid cells. Depending on the source, several types of colonies designated colony forming units and burst forming units develop. Previous reports show the expression of hemoglobin F is induced in those colonies, the burst forming units, which take longer to develop and require higher levels of the hormone erythropoietin. Using a sensitive and specific radioimmunoassay we will study hemoglobin F synthesis and its relation to time, culture conditions and source of culture material and then examine the molecular mechanisms related to the switch.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Our initial objective is to develop a specific and sensitive quantitative assessment of total hemoglobin and fetal hemoglobin synthesis in erythroid cultures. Previous studies have used techniques which provide only relative proportions and not absolute quantities and in addition have required the combination of several colonies, units which although morphologically similar might vary considerably biochemically. Next we would like to correlate the behavior of colony formation in culture to the circulating F cell population in the adult. The final objective will be the use of the culture system and induction of fetal hemoglobin synthesis in burst forming units as a model to approach the molecular events involved in the regulation of the gamma gene locus.

Methods Employed and Major Findings: Erythroid cell cultures have been established using the procedure described by Iscove. A sample of peripheral blood is obtained and the mononuclear cells are then mixed with alpha medium, fetal calf serum, bovine serum albumin, erythropoietin and methylcellulose. This mixture is plated and incubated at 37°C in 5% CO_2 in air for up to two weeks. During this time individual colonies develop, described as burst forming units (BFU-E), from day 10-14 which begin to synthesize sufficient hemoglobin to become red to visual inspection.

We are also using a similar technique on cells derived from samples of bone marrow and in addition to burst forming units, smaller colonies develop at an earlier time which are called colony forming units.

These colonies are being harvested, both individually and by pooling all the colonies on the plate. A radioimmunoassay using tritiated hemoglobin F and specific anti-hemoglobin F antibodies has been developed and is being used to measure hemoglobin F production in the colonies. In a similar manner total hemoglobin production is being measured using another radioimmunoassay.

At the present time culture techniques have been established and are being refined. Preliminary observations using a radioimmunoassay developed in this laboratory have shown appropriate sensitivity and reproducibility to examine gene products in individual erythroid colonies.

Significance to Biomedical Research Tissue culture techniques have provided the means to study cellular regulation removed from the intact organism. The study of hemoglobin synthesis in erythroid culture should provide insight into the mechanism involved in the switch from fetal hemoglobin to hemoglobin A as well as the factors involved in the maintenance of the circulating population of F cells in the adult. Induction of fetal hemoglobin synthesis in individuals with sickle cell anemia or severe forms of thalassemia would have dramatic therapeutic effect if high enough concentrations of hemoglobin F could be attained.

A large amount of information is already available regarding hemoglobin structure and function as well as structural information concerning the genes for the non-alpha globin chain. As such it is one of the most appropriate models to study gene regulation in eukayotic cells.

Proposed Course: Hemoglobin synthesis will be studied in cultures from normal volunteers with regard to colony size and type, time and erythropoietin concentration. At the same time the proportion of fetal hemoglobin and proportion of F cells will be measured in peripheral blood. Similar studies will also be conducted on patients with hemoglobinopathies and thalassemia. The behavior of erythroid cultures derived from fetal tissues will be characterized with respect to the above parameters.

A cDNA probe specific for the gamma gene locus will be used to explore changes in gene structure, nuclease sensitivity and mRNA processing.

No Publications

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 25,033-01 LCB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Enzymatic Resynthesis of Peptide Bonds in Organic Media

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.A. Homandberg	PHS Research Fellow	LCB NIAMDD
	I.M. Chaiken	Research Chemist	LCB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Chemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES X☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to extend capabilities in the study of the effect of amino acid sequence on protein function, a novel enzymatic resynthesis method was applied to several noncovalent fragment complexes. Substitution of water by partially nonaqueous solvents (e.g 90% glycerol) in systems of limited proteolyses, thermodynamically stabilizes the peptide bond and allows resynthesis (30-50%). A noncovalent complex of ribonuclease S-protein and 4-Fl-His^{12}-S-peptide was converted to a covalent form, allowing the study of catalysis at a low pH where the complex would have dissociated. A counterpart system where a peptide, 4-Fl-His^{119} (116-124) was chemically synthesized and complexed to ribonuclease-(1-115) provided additional information to suggest that both His^{12} and His^{119} are general catalytic acid bases. A two-fragment staphylococcal nuclease complex was "restitched" with the exclusion of loop residue, Lys^{49}. This completely abolished activity, suggesting the importance of the integrity of the loop for the correct conformation at the active site. A cytochrome c complex and a prothrombin 1 complex were also restitched, demonstrating the potential for extended studies of covalent hybrids of these proteins.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:
1) To demonstrate an important and relevant point, that peptide bond hydrolyses are not irreversible.
2) To apply an equilibrium perturbation method to different systems of enzymatic-limited proteolysis, to prove objective 1 and show how capabilities of equilibrium perturbation can be applied to proteins of interest.
3) To learn the limitations and practical considerations of this method and to develop general ideas necessary for application elsewhere.
4) To learn more of the molecular architecture which allows restitching (e.g. in most cases loops are involved, how large or small need they be?).
5) To prepare chemically synthesized derivative fragments of altered sequence, to replace them into the complex and to convert the complex to a covalent form so the influence of sequence can be assessed. This differs from the present approach in that a covalent derivative (not necessarily with the same properties of the complex) can be studied.

Methods Employed: Conversion of protein complexes to covalent forms was investigated by SDS-gel electrophoresis. Conventional molecular sieving, ion exchange, paper and affinity chromatography methods were employed for isolation of the products. Characterization involved amino acid analysis, spectral-based substrate assays, and manual sequencing. Peptides were synthesized by the solid phase technique.

Major Findings:
1) 4-Fl-His^{12} Ribonuclease A and 4-Fl-His^{119} Ribonuclease S are inactive; the histidyl residues are both general acid-bases.
2) Staphylococcal nucleases fragment complexes can be converted to des-Lys^{49}-(6-149)-nuclease in the presence of 90% glycerol, stabilizing ligands, and trypsin. This derivative is inactive; therefore the loop region integrity of residues 43-52 is necessary for correct positioning of the active site residues.
3) Practical considerations to use of chemically synthesized, unrefined (unpurified) fragment mixtures as substrates for chemical synthesis; the yield is reduced by less than half as compared to highly purified mixtures and salt (<50 mM) greatly decreases the yield.
4) At least one type of cytochrome c complex can be restitched.
5) The prethrombin 2 + prothrombin fragment 2 complex can be restitched to prethrombin 1, allowing the study of why certain types of these fragments cause certain disease states in blood coagulation.

Significance to Biomedical Research: A better understanding of the role of sequence in protein folding and function will be facilitated by these studies. For instance not only do we now understand that both His^{12} and His^{119} of ribonuclease are equally and interdependently necessary as general acid-bases for catalysis and that the loop region Nature placed near the active site of staphylococcal nuclease is necessary, but in the future we can design molecules with these exemplary points in mind. Perhaps

we can design more efficient molecules with different activities by incorporation of a greater number of more efficient molecular designs than Nature has had time to do up to this point. Enzymatic replacement therapy may be effected by introduction of redesigned synthetic molecules. Or use of fluid dialysis in line with external enzyme reactors containing hyperactive, redesigned enzyme may be possible. The extended prothrombin work will allow the preparation of covalent hybrid derivatives where one of the fragments has been replaced by a fragment liberated from aberrant prothrombin molecule (provided by Nature). Studies of these hybrids will allow us to pinpoint the molecular cause of these prothrombin-related disease states.

Proposed Course of Action:

1) To form covalent prothrombin 1 hybrids and characterize the resultant enzymatic function.

2) To extend restitching to other proteins of relevance where important questions can be answered with this capability.

3) To extend the thermodynamic concepts to other types of systems; e.g. synthesis of oligonucleotides.

No Publications

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

I. HORMONES AND RECEPTORS

Studies on the Structure and Function of Receptors for Glycoprotein Hormones, Bacterial Toxins, and Interferon

Ganglioside modulation of phospholipid components of the lipid bilayer has been shown; these data have been related to specific ganglioside-ligand interactions which induce transmembrane ion fluxes. Ganglioside modulation of the affinity and capacity of the glycoprotein component of these receptors has been shown in artificial bilayer systems. Adrenergic agents have been shown to alter thyroid membrane lipid methylation, and implications for receptor expression have been examined. As a result of chemical modification of the tryptophan residues of the cholera toxin molecule, these residues have been directly linked to the receptor binding site and to subunit dissociation as a result of receptor interactions. The fragment of the α subunit of cholera toxin similar in primary sequence to oxytocin-vasopressin and to the glycoprotein hormone α subunit has been synthesized; its ability to stimulate the adenylate cyclase activity of several membrane systems, independent of NAD or cytosolic factors, appears to validate computer searches of primary sequence relations which defined a structural and functional relationship between glycoprotein hormones and toxins.

. . . . . Drs. G. Lee, D. L. Gill, E. F. Grollman, R. P. Magnusson, E. Consiglio, M. J. S. De Wolf, M. Epstein, N. P. Morris, and L. D. Kohn

Studies on the Mechanism of Tetanus Action

Differences in retrograde transport or receptor binding have been eliminated as a cause of differences in the species specificity of toxicity. Fragments of the toxin have been used to define determinants on the toxin molecule important for receptor recognition. A glycoprotein component of the tetanus toxin receptor has been defined. Toxin modulation of transmembrane Ca^{2+} flux has been shown in vitro; this phenomenon, like toxin effects on membrane $\Delta\Psi$, is the first in vitro action of toxin correlated with the in vivo biological action of the toxin.

. . . . . Drs. N. P. Morris, D. L. Gill, G. Lee, E. F. Grollman, W. H. Habig (Bacterial Toxins Branch, Division of Bacterial Products, Bureau of Biologics, Food and Drug Administration), M. C. Hardegree (Bacterial Toxins Branch), and L. D. Kohn

Studies on Interferon Action

A glycoprotein fragment of the receptor has been isolated which reconstitutes interferon action in vivo. Studies of the mechanism of reconstitution indicate that the process is species-specific and involves binding to or incorporation within the cell.

. . . . . Drs. N. P. Morris, R. M. Friedman (Laboratory of Experimental Pathology, NIAMDD), and L. D. Kohn

Studies on Graves' Disease

Conditions which eliminate the action of those Graves' disease antibodies which inhibit thyrotropin binding have been defined. Calmodulin has been shown to be a component of the membrane important in this phenomena. The thyroglobulin present in the sera of patients with Graves' disease has been shown to be desialated material. SV-40 transformed cloned thyroid cells have been shown to behave like a functioning and nonfunctioning adenomas. An assay for cytotoxic Graves' disease antibodies has been defined and used to evaluate the properties of these gamma globulins.

. . . . . Drs. E. Consiglio, R. P. Magnusson, R. J. Winand (Departement de Clinique et de Semiologie Medicales, Institut de Medecine, Universite de Liege, Belgium), and L. D. Kohn

Studies on Thyroglobulin Biosynthesis and Degradation

The thyroglobulin receptor has been shown to recognize the N-acetylglucosamine moiety of the thyroglobulin carbohydrate moiety. It has been functionally and structurally related to the "Ashwell" receptor on liver membranes despite a different sugar specificity. The thyroglobulin receptor and thyrotropin receptor have been shown to be functionally related. Studies of mannose-phosphate inhibition of receptor function indicate a definite role in biosynthetic and biodegradation processes important in regulating thyroid hormone secretion.

. . . . . Drs. E. Consiglio, J. E. Rall (Clinical Endocrinology Branch, NIAMDD), G. Salvatore (Centro di Endocrinologia ed Oncologia Sperimentale del C.N.R., Naples, Italy), and L. D. Kohn

Insulin Receptors in Genetic Disorders of Insulin Resistance

We have continued to study the genetic components of hormone resistance in fibroblasts cultured from skin biopsies from patients. Fibroblasts in culture have cell surface receptors for polypeptide hormones such as insulin and somatomedins. We have identified the first selective genetic defect of insulin receptors in fibroblasts cultured from a patient with leprechaunoid appearance, intrauterine growth retardation, and clinical insulin resistance. This severe deficit of insulin receptors in conjunction with marked intrauterine growth retardation suggests a major role for insulin and insulin receptors in intrauterine growth.

. . . . . Dr. M. M. Rechler, E. E. Schilling, J. A. Romanus, Dr. C. Grunfeld (Diabetes Branch, NIAMDD), and Dr. A. M. Rosenberg (University of Manitoba, Canada)

Multiplication Stimulating Activity (MSA): An Insulin-Like Growth Factor (Somatomedin) from Cloned Rat Liver Cells

In our studies of the insulin-like growth factor (somatomedin) multiplication stimulating activity (MSA), a polypeptide synthesized by a cloned line of rat liver cells (BRL 3A), progress has been made in the following areas: (i) characterization of a polypeptide biochemically and immunologically related to BRL-MSA and synthesized by explants of fetal rat liver; (ii) demonstration of 20- to 100-fold increased levels of MSA in fetal rat serum; (iii) characterization of growth hormone-dependent binding proteins

for somatomedins in serum of rats and humans; and (iv) direct demonstration that insulin receptors mediate the acute metabolic effects of MSA and insulin on glucose oxidation in adipocytes, whereas the long-term mitogenic effects of MSA and insulin are mediated by receptors different from insulin receptors.

. . . . . Dr. M. M. Rechler, O. Z. Higa, E. E. Schilling, J. A. Romanus, Y. W.-H. Yang, and Dr. A. B. Knight, Jr. (LBP, NIAMDD); Dr. S. P. Nissley, Dr. A. C. Moses, Dr. R. M. White, and P. A. Short (Metabolism Branch, National Cancer Institute); Drs. C. R. Kahn and G. S. King (Diabetes Branch, NIAMDD); Dr. I. Fennoy (Neonatal and Pediatric Medicine Branch, National Institute of Child Health and Human Development); and Dr. H. J. Eisen (Developmental Pharmacology Branch, NICHHD)

Lectin Binding

The binding of peanut agglutinin to model systems consisting of sugars covalently attached to glass beads is being studied. The extent of analogy between the binding of lectin to these systems and the binding of lectins to cell surface carbohydrates is being explored.

. . . . . Drs. H. Flowers (Department of Biophysics, Weizmann Institute, Rehovot, Israel) and A. P. Minton

II. ENZYME MECHANISMS AND PROTEIN STRUCTURE

Tryptophan Synthase of Escherichia coli

The chemistry of the active site of the β_2 subunit of tryptophan synthase is being probed by sequence analysis of specifically modified enzymes. Three previously identified active site residues (a sulfhydryl, a histidyl residue, and a lysyl residue) have all been located in one large cyanogen bromide peptide. The total sequence of this 83-residue peptide is almost complete; location of this peptide relative to the amino terminus of the β chain results in the primary sequence of the first 104 residues of the β chain.

Our previous finding that the α subunit of tryptophan synthase can be specifically cleaved by limited tryptic proteolysis of the $\alpha_2\beta_2$ complex has been extended by separation and characterization of the two proteolytic fragments. The α-1 fragment is the amino terminal fragment (residues 1-188, molecular weight 20,500); the α-2 fragment is the carboxy terminal fragment (residues 189-268, molecular weight 8,300). The separate fragments refold into ordered structures but show no activity or ability to bind the β_2 subunit or the substrate analog indole propanol phosphate. Mixing of the two fragments restores all the properties of the α subunit. The system thus demonstrates the presence of two independently folding domains which are both essential for enzymatic activity.

. . . . . Drs. E. W. Miles and W. Higgins

Aminoacyl tRNA Synthetases

Over a period of years we have accumulated evidence that the glycyl tRNA synthetase of yeast can exist in two forms. These are present in the cells and vary in concentration in a reciprocal manner during growth. The activity of the valyl tRNA synthetase can now be made to oscillate dramatically in vitro, with evidence that the valyl tRNA product exists in two alternating physical forms.

. . . . . Dr. S. Black

Transglutaminase and Tissue Proliferation

Neopentyl-chloroethyl-nitrosourea was found to be a cancerostatic agent in mice because of its releasing neopentylisocyanate, which is an active-site inhibitor of transglutaminase. It is proposed that transglutaminase masks the strangeness of proliferating cells, and when the enzyme is inhibited, the cell's self defenses are eliminated by the cellular immune system.

. . . . . Drs. K. Laki, A. Welebir, and T. Szabo, and S. T. Yancey, Jr. (Laboratory of Chemical Pharmacology, National Cancer Institute)

Interaction of Ions with N-Methylacetamide

The CNDO (complete neglect of differential overlap) method is used to investigate the binding of LiF, LiCl, NaF, and NaCl to N-methylacetamide (NMA) as a model for these ions binding to a peptide moiety. The (cation) ---O=C-N-H--- (anion) interaction is shown to result in a net residual charge on NMA, which becomes more positive as the difference in electronegativity between the anion and cation of the salt present increases. Preliminary studies indicate that salts influence the nuclear magnetic resonance spectrum of N-methylactamide.

. . . . . Drs. S. Abdulnur (Department of Chemistry, American University, Washington, D.C.), J. C. Kertesz, and K. Laki

Hemoglobin and Myoglobin

A mechanism for the desolubilization of sickle hemoglobin by nonaggregating proteins has been formulated.

. . . . . Drs. P. D. Ross (Laboratory of Molecular Biology, NIAMDD) and A. P. Minton

The self-association of cyanmet myoglobin in concentrated solution is being studied by measurement of sedimentation and osmotic equilibria.

. . . . . Drs. M. Hirshfeld (Department of Polymer Research, Weizmann Institute), A. Klarman (Department of Biochemistry, Tel Aviv University, Tel Aviv, Israel), E. Daniel (Department of Biochemistry, Tel Aviv University), and A. P. Minton

Preliminary measurements have been made of oxygen equilibrium in dilute and concentrated solutions of normal and sickle hemoglobin.

. . . . . Dr. A. P. Minton

Reaction of Hemoglobin with Copper and with Hydroxylamine

Although it had been known that copper rapidly oxidizes the β hemes of hemoglobin, it was found that copper will also oxidize the α hemes, but at a rate of only about 20 1/M/sec at pH 6, about a thousandth as fast as for the β hemes. If the rapid oxidation of the β hemes is prevented by reacting the B-93 sulfhydryl groups with mercury or an organic thiol reagent, copper will still oxidize the β hemes, but at a rate about the same as for the α hemes.

Studies on reaction of hydroxylamine with hemoglobin continued. Although the spectral changes during this reaction are similar to those when ferricyanide oxidizes hemoglobin, paramagnetic resonance measurements, and the relatively small decrease in absorption at 630 nm on adding cyanide to the final product

cast some doubt on whether much of the hemoglobin is oxidized by hydroxylamine.

. . . . . Dr. R. B. Simpson

Fibrinogens, Fibrins, and Other Blood Coagulation Factors

Salmon Fibrinogen — Our successful past investigations of factor XIII-mediated crosslinking of salmon fibrin showed other interesting facets. One was the presence of an extremely powerful salmon plasmin, active at temperatures of -20° and lower. It also appeared that the (A)α-chain of salmon fibrinogen was smaller than the (B)β-chain. This is in sharp contrast to the current data that in all fibrinogens the (A)α-chains possess the highest molecular weights. Dansylcadaverine incorporation into α- and γ-salmon fibrin chains via factor XIII gave inconclusive results as to identity and size of α- and β-chains. Simultaneously, the salmon plasminogen was isolated from salmon plasma by affinity chromatography in a high state of purity. The plasmin via urokinase activation was reacted with salmon fibrinogen. Since the (A)α-chain is the most susceptible to plasmin hydrolysis, analysis of this reaction clearly indicated its identity. As visualized by SDS-PAGE, the molecular size of the (A)α-chain is indeed smaller than the (B)β-chain.

. . . . . Dr. J. A. Gladner and P. A. Murtaugh

Lamprey Fibrinogen — Extensive sedimentation-equilibrium studies of lamprey fibrinogen and its isolated (A)α-chain, (B)β-chain, and γ-chain have been performed in 6 M guanidine buffers to ascertain molecular weights. It was found that complete equilibrium was not attained unless longer time segments were utilized. The extended time, however, did not indicate any hydrolysis of these molecules. Molecular weights at 110,000, 75,000, and 50,000 were definitely established for (A)α-chains, (B)β-chains, and γ-chains, respectively. A molecular weight of 350,000 $\pm$ 10,000 for lamprey fibrinogen found in aqueous buffers can only be correlated with the formulation [(A)α, $(B)\beta_2$, γ_2], i.e., a single (A)α-chain for this molecule.

. . . . . Drs. J. A. Gladner, M. S. Lewis, and S. I. Chung

Laser Light Scattering — A laser light scattering technique has been employed for determining the shear modulus of fibrin crosslinked gels. The modulus varied linearly with the number of crosslinks until the tie points between overlapping fibers were saturated. The number of crosslinks required for maximum mechanical strength is very small.

. . . . . Drs. R. J. Nossal and J. A. Gladner

Tissue Culture — The newest phase of investigation involves the isolation of cells which may be utilized to investigate blood coagulation factor XIII (a transglutaminase enzyme) as to (i) mode of biosynthesis and release and (ii) possible involvement in haemostasis. Cells of various origins which might contain factor XIII were screened. An example are endothelial cells from the lining of blood vessels and epithelial cells from liver, lung, and uterus. From preliminary studies epithelial cells from rabbit uterus endometrium were shown to react positively to histochemical techniques employing a fluorescent antifactor XIII (antitransglutaminase). The present emphasis includes: (i) isolation of rabbit uterus cells and the optimization of conditions relative to yield and colonization; (ii) establishment of a finite culture media which includes six hormones thus far to optimize passage at various stages;

and (iii) biochemical characterization. These cells have been maintained in cultural conditions for as long as three months.

. . . . . Drs. J. A. Gladner, H. S. Sun, S. I. Chung, and H. G. Coon

Studies on Modified Fibrinogen

Plasmin Digestion of Citraconylated Fibrinogen — The proteolytic digestion of fibrinogen by plasmin was considerably altered by citraconylation as was demonstrated by disc electrophoretic patterns of the plasmic digests. Fragments D and E usually generated from native fibrinogen were not present; however, higher molecular weight components were obtained. This is similar to observations made on patients with certain abnormal fibrinogens.

Chromatographic Analysis of Methylated Fibrinogen — The highly specific methylation of fibrinogen, via dimethylsulfate modification, resulted in the formation of β-methylaspartic acid and γ-methylglutamic acid derivatives of fibrinogen which were ascertained by chromatographic analysis of the products of a digest resulting from the application of a mixture of enzymes on the modified fibrinogen. Identification of the products of the methylation of fibrinogen by diazomethane and thionylchloride was also obtained by this method.

Structural Analysis of the Methylated Fibrinogen Polymer — The ultrastructure of the methylated fibrinogen, obtained from specific methylation of the carboxylic acid groups by the trimethyloxonium ion, produced a modified fibrinogen which resembled the thrombin-induced physiological clot formation. Evidence of periodicity in the electron micrographs of the methylated fibrinogen was achieved by procedures which generated a low incorporation of methyl groups into fibrinogen. At higher levels of modification the polymerization was less ordered structurally. These results suggest the participation of the carboxyl groups of the α, β, and γ chains of fibrinogen in the organization of the molecular interactions that result in the formation of periodicity during polymer formation.

Demethylation of Modified Fibrinogen — Saponification of the methylated fibrinogen polymer resulted in the solubilization of the modified fibrinogen with the regeneration of native fibrinogen. The action of thrombin on the demethylated fibrinogen produced a fibrin clot with the release of peptides A and B at physiological rates indicating that no denaturation of fibrinogen had occurred.

. . . . . Dr. A. J. Osbahr

Protein Sequences and Structure

Binding Studies and Protein Interactions — Model systems are being developed and studied for explaining cooperative effects in biological macromolecules.

Slow Degradation of Proteins — Sterile solutions of proteins yield peptides and amino acids on standing for various periods of time. A number of proteins are being studied in an attempt to explain this phenomenon adequately. It is proposed that some of the observed activity is intrinsic in the protein molecule itself.

Statistical Analyses of Protein Sequences — The concept of uniqueness and a quantitative method for its evaluation has been developed using Monte Carlo

statistics. Similar methods are being applied to studies on the homology of the immunoglobulins.

. . . . . Dr. H. A. Saroff

Protein Interactions and Conformation

Theoretical studies of the exclusion of proteins from solutions containing polyethylene glycol (PEG) continue. An attractive interaction between PEG and protein has been postulated, and its nature is under investigation.

The effect of particle shape and size upon thermodynamic activity in heterogeneous systems is being theoretically studied. Preliminary results indicate that excluded volume can act as a major determinant of biomacromolecular conformation and quaternary structure in physiological media.

. . . . . Dr. A. P. Minton

III. SYNTHESIS OF PROTEINS AND NUCLEIC ACID

Evolution and Regulation of Genes Involved in Protein Synthesis

Previous studies using a purified mRNA transcript of the tuf gene, which encodes elongation factor Tu, showed directly that duplicate, highly conserved tuf genes are present in Escherichia coli and Salmonella. The almost complete homology between the E. coli and Salmonella tuf genes was surprising in light of the extensive divergence of the remainder of their genomes. We also found that one of the tuf genes (tufA) is contiguous with the fus gene which encodes another peptide chain elongation factor, EF-G. The arrangement of these genes poses an intriguing problem in gene regulation because the tufA gene is cotranscribed with but after the fus gene, and yet the tufA gene product is synthesized at 4 times the rate of the fus gene. In order to pursue both the evolutionary and regulatory studies of the tuf gene, different portions of these genes have been cloned into various plasmid vectors. Using these and other means we have found the following:

(i) Evolutionary Studies — All bacteria examined, except a methanogenic strain, contain DNA sequences of near complete homology to the E. coli tuf gene. Different portions of the E. coli tuf gene are conserved to different extents in the different bacteria, but at least 50% or more of the conserved sequences are homologous to that part of the tuf gene which encodes the carboxy-terminal portion of EF-Tu, which is thought from the work of others to bind the guanine nucleotides that are required for the functioning of EF-Tu. Our finding of these highly conserved sequences was surprising because some of these bacteria are thought to have diverged from their common ancestor with E. coli several billion years ago. Because the genetic code is degenerate and thus DNA sequence need not be conserved to conserve protein sequence, we have concluded that either the tuf sequences have been conserved for reasons other than their protein-coding function or that there exist heretofore unrecognized routes of gene transfer between very diverse prokaryotes. In pursuing this problem we are also determining at what point in the evolution of enteric bacteria that the tuf gene duplication took place. So far, in addition to E. coli and Salmonella, we have detected duplicate tuf genes in Aerobacter, Serratia, and Shigella. By contrast, we have found that

Caulobacter, Myxococcus, Micrococcus, Bacillus, Rhodospirrillium, and Pseudomonas contain only one tuf gene.

. . . . . Drs. D. Filer and A. V. Furano

(ii) Studies on tuf Gene Regulation — In order to determine whether the differential expression of tufA and fus is regulated at the transcriptional or translation level, we set out to determine the relative amounts of mRNA for the tufA and fus genes. We found that the published methods of preparing bacterial RNA suitable for hybridization studies did not yield stable preparations and accordingly devised a new method which results in stable, high molecular weight RNA. Preliminary experiments have shown that cells contain equivalent amounts of tufA and fus gene mRNA, and we have concluded that the differential expression of these genes must take place at the level of translation. This is the first clear example in prokaryotes of a translationally-regulated gene product and should provide an excellent experimental model for understanding the mechanism of this process which appears to be widespread in various higher organisms.

. . . . . Drs. F. S. Young and A. V. Furano

The Mechanism of Action of Nerve Growth Factor (NGF)

We have been studying a cell line (PC12) cloned from a rat pheochromocytoma which responds to NGF by cessation of cell division and differentiation into a cell type with most of the morphological and physiological attributes of a mature neuron. Our previous studies showed that this differentiation is not accompanied by the synthesis of any new major structural polypeptides, and we concluded that the dramatic morphological and physiological alterations exhibited by these cells must take place largely through a rearrangement of the types of major structural protein synthesized by these cells prior to NGF treatment.

To pursue this problem, as well as the more general problem of gene regulation that is attendant upon cessation of cell growth, we have begun to compare the kinds of nuclear RNA transcripts present in the growing (minus NGF) and non-growing (plus NGF) cells. To extend our analysis as well as to examine a source of nuclear RNA from a different cell type, we are also examining nuclear RNA from growing rat hepatocytes (isolated from rat liver remnants, 24 hours after partial hepatectomy) and nongrowing hepatocytes (isolated from sham operated animals). This phase of our problem is just now getting underway, but we have succeeded in devising a very simple and rapid means of obtaining intact total nuclear RNA from all of the above sources.

. . . . . Dr. A. V. Furano

Evolution

The capacity of nucleotide bases to form hydrogen bonds with peptides has been further investigated, and identification made of the interacting groups of peptides and uracil.

. . . . . Drs. C. H. Niu and S. Black

DNA Replication

We have previously shown that the proteins encoded by bacteriophage T4 gene 43 (DNA polymerase), 32 (DNA helix-destabilizing protein), and gene 45, as

well as the complex of the gene 44 and 62 proteins (DNA-dependent ATPase) are all required in vitro for strand displacement synthesis beginning at a nick in duplex DNA. Ribonucleotides are not required for this synthesis. In contrast, the initiation of new chains is dependent upon ribonucleoside triphosphates and two additional phage proteins, the gene 41 product and a protein whose activity is controlled by gene 61.

During the past year we have shown that short ribo-oligonucleotide primers are made by the combination of the gene 41 and 61 proteins. This reaction requires a DNA template and is carried out on all natural single-stranded DNA's tested. Optimal RNA primer and DNA synthesis takes place with all four ribonucleoside triphosphates, but there is substantial primer and DNA synthesis with only rATP and rCTP. Pentanucleotides with a 5' terminal triphosphate are the principal product of the priming reaction. The primers made with γ ^{32}P-rATP have been sequenced by partial digestion with base specific ribonucleases. With rATP and rCTP, the predominant sequence is $pppACCN_4N_5$, with both A and C found in position 4. With all four ribonucleotides the predominant sequence is $pppACN_3N_4N_5$, with G and C and/or U in positions 3 and 4. A similar sequence distribution is found at the 5' terminus of the DNA product.

The RNA primers are made at many different positions along the DNA molecule. Using ϕX174 DNA as a template, we find that there is alkaline phosphatase sensitive, terminal label from γ ^{32}P-rATP in all 11 fragments formed after digestion of the product with HaeIII nuclease. Our working hypothesis is that this priming is an example of the type of initiation required to begin "Okasaki" pieces on the lagging strand during the replication of duplex DNA.

We have been trying to determine whether gene 61 is the structural gene for one of the T4 priming proteins, or encodes a protein required to activate this priming protein. The gene 61 product is made in such small quantities that it cannot be identified by comparing sodium dodecyl sulfate gels of proteins labeled after infection with T4 phages containing or lacking an intact gene 61. We have identified one minor protein band which binds to DNA cellulose during chromatography of 61^+ but not 61^- extracts. We are continuing our efforts to purify the priming protein in order to establish whether the catalytic activity resides in this minor protein band.

The proteins described above initiate only on single-stranded DNA. We have therefore begun a search for additional T4 unwinding or gyrase activities which might be required for initiation within duplex DNA. We have partially purified a protein (or proteins) not present in uninfected cells which catalyzes the relaxation (nicking-closing) of supercoiled DNA.

. . . . . Drs. N. G. Nossal, L. L. Silver, and M. Venkatesan

Methylglyoxal Regulating Gene Transcription

Escherichia coli RNA polymerase was assayed with different nonspecific, specific, and artificial DNAs as templates in the presence and absence of methylglyoxal. The amount of the newly synthesized RNA in the presence of 10^{-8} M methylglyoxal was increased by a factor of 1.5-2.0 using calf thymus or salmon sperm DNAs or poly [d(G-C)] as templates. The poly [d(A-T)] or λ b 511 phage DNA-directed synthesis remained unaffected. The significant stimulation of the RNA synthesis was due to the increase in the number of initiated RNA chains.

. . . . . Drs. S. Damjanovich, J. C. Kertesz, and K. Laki

IV. COMPLEX CARBOHYDRATES

A radioimmunoassay that measures Le^b-active glycolipids in human plasma has been developed using antiserum from a goat immunized with conjugated lacto-N-difucohexaose I. Binding by the antiserum of lacto-N-difucohexaose I conjugated to ^{125}I-labeled bovine serum albumin is specifically inhibited by Le^b-active ceramide hexasaccharide. Plasma levels of the glycolipid are quantitated by comparing the inhibitory activity of plasma with that of the purified Le^b-active glycolipid. Of 35 blood group O plasma samples assayed, only those from $Le(a^-b^+)$ individuals inhibited binding, the average inhibition corresponding to 0.9 µg of Le^b-active ceramide hexasaccharide per ml of plasma (range: 0.2 to 2.5 µg/ml).

. . . . . Drs. V. Ginsburg, D. F. Smith, T. E. Rohr, and C. V. Warner

V. MEMBRANE STUDIES OF ESCHERICHIA COLI AND OF MACROPHAGES

Aldoheptose Biosynthesis and Its Regulation

In earlier collaborative work (with Dr. L. Leive), two classes of E. coli mutants were isolated with altered permeability. These studies have now been extended independently. Genetic analyses had indicated that the genetic alterations involved are distinct in these two mutants. One mutant, designated NbsB, carries a mutation which effects the synthesis of the aldoheptose, L-glycero-D-mannoheptose. It is postulated that L-glycero-D-mannoheptose is formed from D-glycero-D-mannoheptose via an epimeration step. A temperature-sensitive mutant has been isolated with the NbsB phenotype. A crude assay for the epimerase activity and selected hybrid ColE1 plasmids capable of correcting the NbsB phenotype and increasing the epimerase activity 6- to 10-fold relative to the wild type strains, has been developed. This represents the first direct biochemical evidence of aldoheptose biosynthesis.

. . . . . Dr. W. G. Coleman, Jr.

The Outer Membrane of E. coli

We previously isolated a class of E. coli mutants showing increased permeability to a large number of unrelated hydrophobic compounds and mapped it at minute 10 on the E. coli chromosome. Now we find that the sole detectable change in the organism is a loss of 90% of the phosphate in lipid A of lipopolysaccharide (LPS), and revertants losing permeability regain lipid A phosphate. These findings suggest that lipid A is the moiety responsible for hydrophobic impermeability in the outer membrane. We are now studying the hydrophobic permeability of liposomes of bacterial phospholipid, with and without normal and mutant lipid A, to test this hypothesis in an in vitro system.

. . . . . Drs. W. G. Coleman, Jr., and L. Leive

Experiments in this and other laboratories have indicated that the polysaccharide of LPS of the outer membrane may be heterogeneous in length; two or three, or at most, four, size classes have been found in a given strain. Now, using sodium dodecylsulfate gel electrophoresis of very dilute solutions of LPS, we have been able to detect two dozen molecular species in an organism previously thought to have two. These represent different moieties, noncovalently joined, and are probably not multimers of a single species, as

they are stable on rechromatography. This technique will permit more precise understanding of the structure of LPS.

. . . . . Drs. R. C. Goldman and L. Leive

We earlier devised a method for a rapid gentle extraction of LPS from whole cells with aqueous butanol that resulted in a product that was 90-98% LPS plus 2-10% protein. This material has received a great deal of attention from other, immunological, laboratories, as the small amount of protein that copurifies is mitogenic for mutant mice that do not respond to LPS as a mitogen. We have now shown that the proteins that copurify are specifically outer membrane proteins, which should help in the further study of the immunological phenomenon.

. . . . . Drs. D. White, R. C. Goldman, and L. Leive

Macrophage Membranes and Cell Function

We have labeled membranes of macrophage cell lines by borotritide reduction of oxidized sialic acid residues, and defined several labeled protein bands on sodium dodecylsulfate gels, that completely lose their label on sialidase treatment. These proteins shall be used as markers in studies of the specificity of internalization, and rate of entry and recycling, of membrane markers during phagocytosis and pinocytosis.

. . . . . Drs. R. C. Goldman and L. Leive

Mutants of macrophage cell lines that are altered in cell surface components have been selected by isolating mutants resistant to wheat germ agglutinin, deoxyglucose inhibition, and killing by LPS. The biochemical and physiological consequences of these mutations are under study.

. . . . . Dr. L. Leive

VI. MECHANISMS OF INHERITANCE IN SACCHAROMYCES CEREVISIAE

The Killer Virus-Plasmid of Yeast

Background. Two species of double-stranded (ds) RNA, called L (3.0×10^6 daltons) and M1 (1.5×10^6 daltons) are found in K_1 killer strains of S. cerevisiae. Such strains secrete a toxin (coded for by M1) which is lethal only to strains not carrying M1 ds RNA. L codes for the coat protein in which it and probably M1 are encapsulated as virus-like particles. We have previously described three chromosomal genes whose products are essential for expression of the information in M1 ds RNA (kex and rex genes), 28 chromosomal genes needed for maintenance or replication of M but not for L (26 mak genes, pet18, and spe2), and 6 genes with regulatory effects on K_1 plasmid replication (4 ski genes, KRB1, and a mitochondrial gene). A plasmid-coded replication gene (sd) was also described.

S. cerevisiae strains with a second killer specificity, called K_2, have also been described. The K_2 killer trait is also due to a non-Mendelian genetic element, called [KIL-k_2], which has been identified as a 1.0×10^6 dalton ds RNA molecule (M_2).

New Findings. [KIL-k_2] depends on at least mak16 and pet18 for its replication. In studying the inheritance of the K_2 killer plasmid [KIL-k_2], we have

discovered two new plasmids, called [EXL] and [NEX], and two new chromosomal genes, called mkt1 and man1. The [EXL] plasmid has the ability to prevent the replication of the [KIL-k_2] plasmid when both are together in a cell. This property of [EXL] is called exclusion. The [NEX] plasmid prevents [EXL] from excluding [KIL-k_2] when all three plasmids are introduced into the same cell. [NEX] is so named because it makes [KIL-k_2] nonexcludable.

The chromosomal MKT1 gene is needed for maintenance of [KIL-k_2], if, and only if, the [NEX] plasmid is present. That is, a strain lacking [NEX] does not need the MKT1$^+$ product in order to maintain [KIL-k_2], but a strain carrying [NEX] does need MKT1$^+$ to replicate [KIL-k_2]. This is rather paradoxical because [NEX] helps retain [KIL-k_2] by preventing [EXL] action, but, when the cell is mkt1$^-$, [NEX] eliminates [KIL-k_2] even in the absence of [EXL].

The chromosomal MAN1 gene is simply a gene needed for the maintenance or replication of the [NEX] plasmid. It is not needed for the maintenance of either [KIL-k_2] or [EXL].

"Defective-interfering virus" mutants of [KIL-k_1] are deletion mutants of part of the M_1 ds RNA which interfere with replication of the normal M_1 ds RNA. In a ski2-2 host, we have found that this interference does not occur. This effect is, in part, due to the induction by the ski2-2 mutation of a mutation in a plasmid-coded replication function.

Using cytoduction (heterokaryon formation) strains useful for *sake* brewing were constructed.

. . . . . Drs. R. B. Wickner, A. Toh-e, P. Guerry-Kopecko, and K. Ouchi

VII. POLYAMINES

Mutations in E. coli and S. cerevisiae Resulting in Polyamine Deficiency and Studies on the Biosynthetic Enzymes

We have extended our studies with the amine-deficient mutants of E. coli which we have constructed. These mutants are completely deficient in putrescine and spermidine as a result of deletion mutations in all four genes used for polyamine biosynthesis; namely, the genes for ornithine decarboxylase, arginine decarboxylase, agamatine ureohydrolase, and adenosylmethionine decarboxylase. The growth rate of these quadruple mutants is markedly slowed; the doubling time is 5 hours. These cells, however, are able to maintain bacteriophages T4, T7, and f2, and the burst size of these phages is about the same in deficient and supplemented cultures. Two important defects in function have been observed. (i) Amine-deficient quadruple mutants cannot produce viable λ bacteriophage, either in a lytic infection, or after induction of a lysogenic derivative. λ lysogeny, however, is maintained normally in the absence of amines. (ii) Matings with an amine-deficient Hfr donor have a 300-fold reduction in the number of recombinants, even though this mutant can transfer its chromosome to a recipient at a normal rate. Also, adsorption of bacteriophage f2 is markedly less efficient in the amine-deficient organism, even though the burst size is normal. We are studying the mechanisms of these defects as part of our studies on the specific functions of the polyamines in E. coli.

In order to study further the pyruvate cofactor of E. coli adenosylmethionine decarboxylase, we have isolated a plasmid-containing strain from the Carbon

collection which carries the E. coli gene for the adenosylmethionine decarboxylase. We have developed culture conditions for cells carrying this plasmid to amplify the amount of enzyme that we can obtain for study and have been purifying the enzyme from this source.

We are isolating and mapping mutants of E. coli in lysine decarboxylase in order to eliminate the formation of cadaverine from lysine in the polyamine-deficient organism. We have found evidence for two genes, mapping at different locations; one the gene for the structural protein, the other a control gene.

. . . . . Drs. E. W. Hafner, C. W. Tabor, and H. Tabor

Yeast ornithine decarboxylase is of particular interest as it is a convenient example of the eukaryote enzyme. We have been isolating mutations in ornithine decarboxylase in yeast in order to obtain yeast strains that are completely deficient in putrescine as well as spermidine and spermine. We have shown that such strains have an absolute requirement for an amine for growth. Several bypass mutants of these deficient strains have been obtained, and these are being studied to determine the factors controlling eukaryotic polyamine biosynthesis. At least two genes involved in ornithine decarboxylase synthesis have been obtained; one clearly is a regulatory gene. As part of these studies we have shown that ornithine decarboxylase is controlled by spermine and/or spermine synthase. We have also shown that yeast has two separate propylamine transferase enzymes, one for spermidine synthesis and one for spermine synthesis.

We have also been purifying yeast ornithine decarboxylase from a mutant strain which overproduces this enzyme.

Preliminary studies have been carried out on cloning the yeast gene for adenosylmethionine decarboxylase in E. coli in order to prepare large amounts of this enzyme for study and to study its regulation.

. . . . . Drs. M. S. Cohn, C. W. Tabor, and H. Tabor

An E. coli strain carrying a plasmid containing the structural gene for adenosylmethionine synthetase has been isolated and used for the purification of large amounts of this enzyme. The mechanism of action of this enzyme and the kinetics of the reaction have been studied.

. . . . . Drs. G. D. Markham, E. W. Hafner, C. W. Tabor, and H. Tabor

Changes in Ornithine Decarboxylase as Assay for Histocomptability

We have devised a rapid assay for mixed leucocyte histocompatibility, based on the very early striking increases in ornithine decarboxylase and adenosylmethionine decarboxylase, two of the enzymes involved in polyamine biosynthesis. We have also developed an even more rapid assay based on an acetylation reaction. We have obtained a highly purified growth factor from regenerating liver, which acts specifically on liver cells in culture. We have continued our studies of rat liver adenosylmethionine decarboxylase.

. . . . . Drs. A. A. Demetriou, C. W. Tabor, and H. Tabor

VIII. TRAUMATIC SHOCK AND CELLULAR IMMUNITY

Continued work on the defect in cellular immunity produced by extensive burns

in mice has shown no significant impairment in the migration of peritoneal lymphocytes but a decreased migration of splenic lymphocytes. No decrease in lymphokine production was found.

The effect of catecholamines and adrenergic blocking agents on shock mortality was studied in mice, following burn and tourniquet trauma and endotoxin. Epinephrine and norepinephrine increased mortality given as pretreatment and had no effect given after trauma. Propanolol decreased mortality as pretreatment but was ineffective later. Dibenamine lowered mortality from endotoxin shock. None of these agents prevented fall in body temperature.

Experiments were continued on the relation of sodium chloride administered in the diet of normal mice on fluid movement, blood pressure, and longevity.

. . . . . Drs. K. Markley (deceased) and S. M. Rosenthal and Mrs. E. T. Smallman

IX. MYCOBACTERIUM

Continued studies on the growth of megakaryocytes revealed a very fine structure of the demarcation membrane system of the cell, a phenomenon which has not yet been observed in cultures.

Motion picture studies on the growth of megakaryocytes showed various stages of maturation of the cells, including endomitosis, marked motility, and formation of the demarcation membrane system, followed by extrusion of numerous cytoplasmic projections suggesting the phenomenon of platelet liberation.

. . . . . Dr. Y. T. Chang

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 23,140-21 LBP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biochemistry of Sulfur-Containing Compounds

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Simon Black, Ph.D.	Biochemist	LBP NIAMDD
	and Chief, Section on Biochemistry of Amino Acids		LBP NIAMDD
OTHER:	Chien-Hua Niu, Ph.D.	Staff Fellow	LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
Section on Biochemistry of Amino Acids

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

I. Aminoacyl tRNA synthetase regulation. During the oscillating activity of a valyl tRNA synthetase from yeast the valyl tRNA product has been indicated to occur in two alternating physical forms.

II. Evolution. Hydrogen bonds between the uracil ring and peptides have been identified as occurring in pairs that are equal in strength to the pair of bonds formed under comparable conditions between uracil and adenine.

PHS-6040
(Rev. 10-76)

Project Description:

I. Protein Synthesis. The aminoacyl tRNA synthetases of all living organisms catalyze the following general reaction:

amino acid + ATP + tRNA $\rightleftharpoons$ aminoacyl tRNA + AMP + PP.

We have found previously that within the yeast cell two forms of the glycyl tRNA synthetase co-exist, one being dependent on activator-like substances, whereas the other is active in the absence of any special additives. These two forms appear to be cyclically interconverted during growth as part of a regulatory mechanism. More recently the activity of the valyl tRNA synthetase of yeast was found to oscillate in vitro under certain conditions. During the past year this phenomenon has been studied intensively, and it is now possible to produce dramatic oscillations in which the concentration of valyl tRNA rises and falls between extreme values, with indications that this product exists alternately in two very different physical forms. This appears to be a phenomenon of a type heretofore unknown in enzymology.

We plan to continue this study with the general objective of elucidating the detailed components and operation of the mechanism of this regulatory phenomenon in the first step of protein synthesis.

II. Evolution. Experiments designed to elucidate the origin of the genetic code have been continued. During the past year the previous demonstration of hydrogen bond formation between uracil and peptides has been further developed. A pair of bonds, equivalent in strength to the comparable pair between uracil and adenine, forms between the N(3)H and C(4)=O of the base and the C=O and N-H, respectively, of each aminoacyl residue of a peptide. Peptide-adenine bonds are about one-third as strong, and peptide-guanine bonds one-tenth as strong as those between peptide and uracil. Experiments are now underway to test the binding of cytosine derivatives to peptides, particularly to peptides of serine and threonine, which could throw important light on the origin of codons with cytosine as the central base.

Publications:

Niu, C.-H., and Black, S.: Hydrogen bonding in solution between the uracil ring and the peptide backbone demonstrated by nuclear magnetic resonance spectroscopy. J. Biol. Chem. 254: 265-267, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 23,230-29 LBP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Chemotherapy of Mouse Leprosy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Yao Teh Chang, M.D. Research Pharmacologist LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Continued studies on the growth of megakaryocytes revealed a very fine structure of the demarcation membrane system of the cell, a phenomenon which has not yet been observed in cultures.

Motion picture studies on the growth of megakaryocytes showed various stages of maturation of the cells, including endomitosis, marked motility, and formation of the demarcation membrane system, followed by extrusion of numerous cytoplasmic projections suggesting the phenomenon of platelet liberation.

1-6040
Rev. 10-76)

Project Description:

Growth of Megakaryocytes in Cultures

The appearance of the coarse demarcation membrane system of megakaryocytes was reported last year. The fine structure of the demarcation membrane system is now being observed in the cells. This phenomenon was observed only by frequent sampling of cells within the first 24 hours of cultivation. It showed numerous, very fine cytoplasmic lines (approximately the width of platelets) within the cytoplasm, with nuclear materials appearing at regular intervals, suggesting the formation of chains of platelets. Long, thin cytoplasmic projections with regular areas of constrictions suggested the appearance of the liberation of platelets from the cells. Such phenomenon has been observed by electron microscopy but not yet by light microscopy in stained specimens of cultured megakaryocytes.

The fine demarcation membrane system was not observed at the later stages of maturation of megakaryocytes in the cultures. Instead of forming the fine demarcation membrane system, the coarse demarcation membrane system terminated by generalized degranulation of the cytoplasm, leading to formation of ghost cells with an empty cytoplasm containing large masses of lobulated nucleus.

Motion picture studies on the growth of megakaryocytes revealed various steps of endomitosis, granulation of cytoplasm, massive liberation of platelets, and numerous cytoplasmic projections in various configurations. Prior to the appearance of cytoplasmic projections, there was a stage of active movement of the cells with marked changes of their morphological appearances.

Although the maturation process of megakaryocytes was not complete in the present study, the life cycle of the cell may be constructed by combination of the coarse demarcation membrane system observed at the later stages and the fine demarcation membrane system at the beginning. The cycle is believed to be as follows:

The small blast-like cells (megakaryoblasts) first showed several successive endomitosis with corresponding increase in the size of the cytoplasm. Granulation appeared in the cytoplasm. A period of rapid movement of cells was observed at this time, followed first by formation of the coarse and then the fine demarcation membrane systems. Liberation of platelets appeared either by massive bursts of the cytoplasm or by segmentation of the long, thin cytoplasmic projections. Finally, the cells revealed numerous cytoplasmic projections in various types of configurations. Death of cells was shown by the appearance of large bubbles in the cytoplasm.

Visit to the People's Republic of China

I visited a leprosy hospital in Shanghai during a visit to the People's Republic of China in 1978. It appears that I was the first foreign person permitted to visit a leprosy hospital in China since its establishment in 1949. There are 3,800 leprosy cases in a population of 10×10^6 in that area. There is one hospital and a number of leprosy stations and leprosy

villages located in various communes. The barefoot doctors are trained to locate new cases and treat the out-patients. The total medical staff is 175, a staff/patient ratio outnumbering by far that of other countries, perhaps with the exception of Carville in the United States. All the western anti-leprosy drugs, including the compound B663, are used there. There is one herb drug believed to be as effective in the treatment of lepra reaction as the standard drug, phthalidomide. While I was in China, I was invited to give three lectures at various medical institutions, including the leprosy hospital.

Publications:

Chang, Y. T.: Leprosy control in Shanghai, China. (Editorial) Int. J. Lepr. 47: 56-58, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 23,330-01 LBP

PERIOD COVERED
April 22, 1979 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Aldoheptose Biosynthesis and Its Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William G. Coleman, Jr., Ph.D. Research Microbiologist LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two classes of Escherichia coli mutants have been isolated with altered permeability. Genetic analyses indicated that the genetic alterations involved are distinct in these two mutants. One mutant, designated NbsB, carries a mutation which effects the synthesis of the aldoheptose, L-glycero-D-mannoheptose. It is postulated that L-glycero-D-mannoheptose is formed from D-glycero-D-mannoheptose via an epimeration step. A temperature-sensitive mutant has been isolated with the NbsB phenotype. A crude assay for the epimerase activity and selected hybrid ColE1 plasmids capable of correcting the NbsB phenotype and increasing the epimerase activity 6- to 10-fold relative to the wild type strains, has been developed. This represents the first direct biochemical evidence of aldoheptose biosynthesis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

(1) To elucidate and characterize the biochemical steps leading from sedoheptulose-7-P to L-glycero-D-mannoheptose. (2) To study the regulatory mechanisms involved in these steps. (3) To isolate and purify the proteins involved in aldoheptose biosynthesis.

Major Findings:

(1) A mutant of E. coli K-12 altered in the final step of aldoheptose biosynthesis has been isolated and genetically and biochemically characterized.

(2) Temperature-sensitive mutants with genetic alterations which affect aldoheptose biosynthesis have been isolated.

(3) Hybrid ColE1 plasmids capable of complementing the aldoheptose deficiency have been selected. In addition, strains carrying these plasmids produce 6- to 10-fold more enzyme than the parent strains.

Publications:

Coleman, W. G., Jr., and Leive, L.: Two mutations which affect the barrier function of the Escherichia coli K-12 outer membrane. J. Bacteriol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 23,580-16 LBP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Protein Synthesis and Gene Expression in Escherichia coli and Other Organisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Anthony V. Furano, M.D.	Medical Officer (Research)	LBP NIAMDD
OTHERS:	David Filer, Ph.D.	Visiting Fellow	LBP NIAMDD
	Fredric S. Young, Ph.D.	Staff Fellow	LBP NIAMDD

COOPERATING UNITS (if any)

Dr. Lloyd Greene, Department of Pharmacology, New York University Medical School, New York, New York

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SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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3.6	2.8	0.8

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are continuing our investigations into the regulation of protein synthesis and gene expression in Escherichia coli. In addition, we have extended our studies to examine the mechanism whereby nerve growth factor (NGF) induces the differentiation of immature neural cells into mature neurons. The studies on NGF are being carried out with the collaboration of Dr. Lloyd Greene.

PHS-6040
(Rev. 10-76)

Project Description:

I. Evolution and Regulation of Genes Involved in Protein Synthesis

Previous studies using a purified mRNA transcript of the tuf gene, which encodes elongation factor Tu, showed directly that duplicate, highly conserved tuf genes are present in Escherichia coli and Salmonella. The almost complete homology between the E. coli and Salmonella tuf genes was surprising in light of the extensive divergence of the remainder of their genomes. We also found that one of the tuf genes (tufA) is contiguous with the fus gene which encodes another peptide chain elongation factor, EF-G. The arrangement of these genes poses an intriguing problem in gene regulation because the tufA gene is cotranscribed with but after the fus gene, and yet the tufA gene product is synthesized at 4 times the rate of the fus gene. In order to pursue both the evolutionary and regulatory studies of the tuf gene, different portions of these genes have been cloned into various plasmid vectors. Using these and other means we have found the following:

(a) Evolutionary Studies — All bacteria examined, except a methanogenic strain, contain DNA sequences of near complete homology to the E. coli tuf gene. Different portions of the E. coli tuf gene are conserved to different extents in the different bacteria, but at least 50% or more of the conserved sequences are homologous to that part of the tuf gene which encodes the carboxy-terminal portion of EF-Tu, which is thought from the work of others to bind the guanine nucleotides that are required for the functioning of EF-Tu. Our finding of these highly conserved sequences was surprising because some of these bacteria are thought to have diverged from their common ancestor with E. coli several billion years ago. Because the genetic code is degenerate and thus DNA sequence need not be conserved to conserve protein sequence, we have concluded that either the tuf sequences have been conserved for reasons other than their protein-coding function or that there exist heretofore unrecognized routes of gene transfer between very diverse prokaryotes. In pursuing this problem we are also determining at what point in the evolution of enteric bacteria that the tuf gene duplication took place. So far, in addition to E. coli and Salmonella, we have detected duplicate tuf genes in Aerobacter, Serratia, and Shigella. By contrast, we have found that Caulobacter, Myxococcus, Micrococcus, Bacillus, Rhodospirrillium, and Pseudomonas contain only one tuf gene.

(b) Studies on *tuf* Gene Regulation — In order to determine whether the differential expression of tufA and fus is regulated at the transcriptional or translation level, we set out to determine the relative amounts of mRNA for the tufA and fus genes. We found that the published methods of preparing bacterial RNA suitable for hybridization studies did not yield stable preparations and accordingly devised a new method which results in stable, high molecular weight RNA. Preliminary experiments have shown that cells contain equivalent amounts of tufA and fus gene mRNA, and we have concluded that the differential expression of these genes must take place at the level of translation. This is the first clear example in prokaryotes of a translationally-regulated gene product and should provide an excellent experimental model for understanding the mechanism of this process which appears to be widespread in various higher organisms.

II. The Mechanism of Action of Nerve Growth Factor (NGF)

We have been studying a cell line (PC12) cloned from a rat pheochromocytoma which responds to NGF by cessation of cell division and differentiation into a cell type with most of the morphological and physiological attributes of a mature neuron. Our previous studies showed that this differentiation is not accompanied by the synthesis of any new major structural polypeptides, and we concluded that the dramatic morphological and physiological alterations exhibited by these cells must take place largely through a rearrangement of the types of major structural protein synthesized by these cells prior to NGF treatment.

To pursue this problem, as well as the more general problem of gene regulation that is attendant upon cessation of cell growth, we have begun to compare the kinds of nuclear RNA transcripts present in the growing (minus NGF) and nongrowing (plus NGF) cells. To extend our analysis as well as to examine a source of nuclear RNA from a different cell type, we are also examining nuclear RNA from growing rat hepatocytes (isolated from rat liver remnants, 24 hours after partial hepatectomy) and nongrowing hepatocytes (isolated from sham operated animals). This phase of our problem is just now getting underway, but we have succeeded in devising a very simple and rapid means of obtaining intact total nuclear RNA from all of the above sources.

Publications:

Furano, A. V.: Direct demonstration of duplicate tuf genes in enteric bacteria. Proc. Natl. Acad. Sci. U.S.A. 75: 3104-3108, 1978.

Furano, A. V., and Sullivan, M.: The peptide chain elongation factor genes tufA and fus of Escherichia coli are intimately related physically. J. Biol. Chem. 253: 8351-8354, 1978.

McGuire, J. C., Greene, L. A., and Furano, A. V.: NGF stimulates incorporation of fucose or glucosamine into an external glycoprotein in cultured rat PC12 pheochromocytoma cells. Cell 15: 357-365, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 23,630-14 LBP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biology of Complex Carbohydrates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor Ginsburg, Ph.D.	Research Chemist	LBP NIAMDD
	and Chief, Section on Biochemistry		LBP NIAMDD
OTHERS:	David F. Smith, Ph.D.	Senior Staff Fellow	LBP NIAMDD
	Thomas E. Rohr, Ph.D.	Staff Fellow	LBP NIAMDD
	Concepcion V. Warner, Ph.D.	Staff Fellow	LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.4	4.0	1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Oligosaccharides will be isolated from natural sources. Oligosaccharides will be coupled to macromolecules in order to make antibodies specific for certain sugar sequences. The antibodies will be used in radioimmune assays for the occurrence of these sugar sequences in the glycolipids and glycoproteins of cell membranes and serum in order to investigate the involvement of the complex carbohydrates of cell surfaces in development and regulation or as tumor-specific antigens.

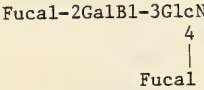
Waldenström macroglobulins and myeloma proteins will be screened for their ability to react with human erythrocytes. Those that do will be tested for their ability to react with various oligosaccharides coupled to macromolecules in order to define their specificity.

PHS-6040
(Rev. 10-76)

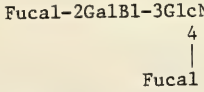
Project Description:

Many carbohydrates of cell membranes occur at levels too low to be detected by conventional means. We are developing immunological methods for their detection using two general approaches for obtaining carbohydrate-specific antibodies: (1) animals will be immunized with pure oligosaccharides coupled to macromolecules and (2) the specificities of Waldenström macroglobulins and myeloma proteins which bind carbohydrates will be determined.

The Le^b blood group antigen is a glycolipid with the terminal carbohydrate sequence:



The same terminal sequence is found in lacto-N-difucohexaose I, an oligosaccharide found in human milk:



Antiserum from a goat immunized with lacto-N-difucohexaose I conjugated to polylysine specifically binds the Le^b carbohydrate sequence [Zopf, D. A., Ginsburg, A., and Ginsburg, V. (1975) J. Immunol. 115: 1525-1529; Zopf, D. A., Ginsburg, V., Hallgren, P., Jonsson, A.-C., Lindberg, B. S., and Lundblad, A. (1979) Eur. J. Biochem. 93: 431-435]. A radioimmunoassay that measures Le^b-active glycolipids in human plasma has been developed using this antiserum. Binding by the antiserum of lacto-N-difucohexaose I conjugated to ^{125}I-labeled bovine serum albumin is specifically inhibited by Le^b-active ceramide hexasaccharide. Plasma levels of the glycolipid are quantitated by comparing the inhibitory activity of plasma with that of the purified Le^b-active glycolipid. Of 35 group O plasma samples assayed, only those from $Le(a^-b^+)$ individuals inhibited binding, the average inhibition corresponding to 0.9 µg of Le^b-active ceramide hexasaccharide per ml of plasma (range: 0.2 to 2.5 µg/ml); no Le^b-active glycolipid (less than 0.02 µg/ml) could be detected in plasma from blood group O individuals of group $Le(a^+b^-)$ or $Le(a^-b^-)$. Plasma from $Le(a^-b^+)$ individuals belonging to blood group A_2 contained more Le^b-active glycolipid than plasma from $Le(a^-b^+)$ individuals belonging to blood group A_1: its level in 19 samples of A_1 plasma averaged 0.2 µg/ml (range: 0.1 to 0.45 µg/ml), while its level in 9 samples of A_2 plasma averaged 1.1 mg/ml (range: 0.8 to 1.3 µg/ml).

The difference in levels of glycolipid in A_1 and A_2 plasma can be explained by competition between glycosyltransferases for a common precursor of the A and Le^b blood group antigens. Because A_1 individuals form more A antigen than A_2 individuals, A_1 individuals would be expected to form less Le^b antigen than A_2 or O individuals.

Coupling of ß-(p-aminophenyl)ethylamine derivatives of sialyloligosaccharides to protein via their phenylisothiocyanate intermediates proceeds under mild conditions, and labile sugar linkages are preserved. Conjugates of bovine serum albumin containing 10-40 mols of oligosaccharide per mol of protein have been prepared with the following oligosaccharides: 3'-sialyllactose, NeuNAcα2-3Galß1-4Glc; 6'-sialyllactose, NeuNAcα2-6Galß1-4Glc; LS-tetrasaccharide c, NeuNAcα2-6Galß1-4GlcNAcß1-3Galß1-4Glc; LS-tetrasaccharide b, Galß1-3[NeuNAcα2-6]GlcNAcß1-3Galß1-4Glc; and disialyllacto-N-tetraose, NeuNAcα2-3Galß1-3[NeuNAcα2-6]GlcNAcß1-4Glc. Rabbits immunized with these synthetic glycoproteins produce antibodies directed against the oligosaccharides. The antibodies can distinguish various sialic acid linkages in complex carbohydrates.

Proposed Course:

Oligosaccharides will be isolated from natural sources. Certain oligosaccharides will be coupled to macromolecules in order to make antibodies specific for certain sugar sequences. The antibodies will be used in radioimmune assays for the occurrence of these sugar sequences in the glycolipids and glycoproteins of cell membranes and serum.

Waldenström macroglobulins and myeloma proteins will be screened for their ability to react with human erythrocytes. Those that do will be tested for their ability to react with various oligosaccharides coupled to macromolecules in order to define their specificity.

Publications:

Ginsburg, V., McGinniss, M. M., and Zopf, D. A.: Biochemical basis for some blood groups. In Sandler, S. G., Nusbacher, J., and Schanfield, M. S. (Eds.): Immunobiology of the Erythrocyte, Progress in Clinical and Biological Research Series. New York, Alan R. Liss, Inc., 1979, in press.

Rohr, T. E., Smith, D. F., Zopf, D. A., and Ginsburg, V.: Le^b-active glycolipid in human plasma: measurement by radioimmunoassay. Arch. Biochem. Biophys., in press.

Zopf, D. A., Ginsburg, V., Hallgren, P., Jonsson, A.-C., Lindberg, B. S., and Lundblad, A.: Determination of Le^b-active oligosaccharides in urine of pregnant and lactating women by radioimmunoassay. Eur. J. Biochem. 93: 431-435, 1979.

Zopf, D. A., Tsai, C.-M., and Ginsburg, V.: Antibodies against oligosaccharides. In Goldstein, I. J. (Ed.): Carbohydrate-Protein Interaction. Am. Chem. Soc. Symposium Series No. 88, 1979, pp. 90-101.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 23,730-19 LBP (formerly: Z01 AM 31,000-18 LBC)

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Factors Involved in Blood Coagulation and Related Fields

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jules A. Gladner, Ph.D.	Research Chemist	LBP NIAMDD
OTHERS:	Ralph J. Nossal, Ph.D.	Guest Worker	LBP NIAMDD
	Hee Sik Sun, M.D.	Fogarty International Postdoctoral Fellow	LBP NIAMDD
	Patricia A. Murtaugh	Chemist	LBP NIAMDD

COOPERATING UNITS (if any)
Soo Il Chung, Ph.D., Senior Investigator, LB, NIDR
Marc S. Lewis, Ph.D., Senior Investigator, BEIB, DRS
Hayden G. Coon, Ph.D., Senior Investigator, LCB, NCI

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.8	3.3	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tissue culture is being utilized to investigate biosynthesis and regulation of coagulation factor XIII. Highly active salmon plasmin has been isolated and utilized to illustrate that the (A)α-chain of salmon fibrinogen is smaller than the (B)β-chain. Extensive sedimentation-equilibrium analysis of lamprey fibrinogen and its (A)α-chain, (B)β-chain, and γ-chain gives molecular weights indicating the formulation [(A)α, (B)β_2, γ_2]. A noninvasive technique, laser light scattering, was utilized to correlate the rigidity of noncrosslinked and crosslinked fibrin clots. The fibrin gel modulus was found to vary linearly with the number of crosslinks until the points between overlapping fibers were saturated.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The final phases of protein-protein interactions in the "cascade scheme" of blood coagulation lead to fibrin formation via thrombin action. Fibrin clots are then stabilized by isopeptide bonds (cross-linking) formed between fibrin monomers mediated by coagulation Factor XIII. These biomolecular reactions have been examined in lower vertebrates in order to elucidate possible differences and/or similarities in molecular structure and action which may be useful in understanding and treating disorders in man. Additionally, Factor XIII and like transglutaminases may play a major role in other processes where protein stabilization through cross-linking are implicated such as wound healing, conception, etc. Investigations have now been initiated as to the biosynthesis, regulation, storage and release of Factor XII in tissue cultures, i.e., dynamic systems.

Methods:

All applicable methodology of protein and enzyme isolation, purification and characterization are employed. Physical biochemistry includes ultracentrifugation and laser light scattering. In conjunction with tissue culture studies radioactive assays include incorporation and radioactive immuno assays. Fluorescent antibody (FITC) techniques are also utilized.

Resume of Ongoing Investigations:

I. Tissue Culture.

The feasibility of utilizing cell cultures to study the biosynthesis and regulation of Factor XIII has been initiated. Cells of various organs and tissue were screened, such as endothelial cells from blood vessel linings, epithelial cells from liver (hepatocytes), lung, and uterus. Epithelial cells from rabbit uterus endometrium have proven to be the most promising. By histochemical techniques employing fluorescent Antifactor XIII (anti-transglutaminase from goat by rabbit Factor XIII) positive reactions of rabbit uterus slices and uterine epithelial cells were obtained. The best method for obtaining the intact epithelial cells has been via digestion of the sterile everted uteri horns by a mixture of crude trypsin and collagenase. Modified Ham's F-12 culture media was employed containing .5% fetal calf serum and six growth factors, insulin, hydrocortisone, transferrin, GHL, somatostatin and thyrotropin. Effects of other hormones, cyclic AMP and DES on propagation were negative. Pretreatment of petri dishes with fibronectin was extremely effective for promoting attachment of the cells. The propagation rate of the uterine epithelial cells is very slow although they have been maintained in cultural condition up to three months. Biochemical characterization has been initiated starting with ^{3}[H]-thymidine and ^{14}[C]-L-leucine uptake. Other tropic hormones and methods are being investigated to develop this cell line.

Drs. J.A. Gladner, H.S. Sun, S.I. Chung and H.G. Coon.

II. Fibrinogens, Fibrins and Other Coagulation Factors. Salmon Fibrinogen Fibrin and Plasmin.

We have demonstrated in the past that coagulation Factor XIII would cross-link salmon fibrin chains to γ-dimers, but left doubt as to which, the α- or β-chain was being cross-linked to the higher polymer. Usually Factor XIII cross-links γ- and α-chains. The molecular size of the α- and β-chains of this species of fibrinogen and fibrin were not resolved; usually (A)α, and α-chains, respectively are the largest. An attempt to identify the α- and γ-chains of salmon fibrin by dansyl cadaverine incorporation via Factor XIII gave inconclusive results. This dilemma was solved by another experimental pathway. As we reported, salmon plasma contains an extremely powerful plasmin, active at temperatures of -20°C and lower. Utilizing DFP treated salmon plasma we were able to isolate this plasminogen by affinity chromatography on columns of lysine-sepharose 4B in highly purified form. This zymogen was activated by urokinase to the active plasmin. Highly purified salmon fibrinogen was obtained as part of this procedure. In all cases investigated to date, the (A)α-chain of fibrinogen is the first (and most) susceptible to plasmin degradation. When the salmon fibrinogen was reacted with the active plasmin, the (A)α-chain could be easily identified on SDS-PAGE gels and proved to be smaller in size than the (B)β. Factor XIII cross-linking experiments showed (on disc gels) that the band which was the most susceptible to plasmin degradation was identical to that which cross-linked to a polymer greater in size to the identifiable γ-dimer. This is good evidence that (A)α-chains of salmon fibrinogen are of smaller size than (B)β's. Salmon plasminogen and plasmin are under detailed study.

Dr. J. A. Gladner and P. A. Murtaugh

III. Lamprey Fibrinogen.

The structure of lamprey fibrinogen has been the object of prolonged investigation. In aqueous solutions, sedimentation-equilibrium analyses constantly yield a molecular weight of 350,000±10,000. Fibrinogens are proposed to consist of 3 species of chains formulated [$(A)\alpha_2$, $(B)\beta_2\gamma_2$]. Molecular weights of 110,00, 78,000, and 50,000 have been deduced for the (A)α, (B)β, and γ chains, respectively by analysis on SDS-polyacrylamide gels, chromatography on sepharose in 6 M guanidine and sedimentation-equilibrium in 6 M guanidine. If the conventional formulation is utilized to calculate the molecular weight, the results give a mass of 470,000, far in excess found by us via sedimentation equilibrium. However, the data are in complete agreement with the formulation [$(A)\alpha$, $(B)\beta_2$, γ_2] equal to 360,000, i.e., a single (A)α entity for this premative fibrinogen. In order to discern the possibility of hydrolysis during centrifugation, extensive studies of lamprey fibrinogen and its isolated chains were performed in 6 M guanidine buffers for extended times. A total of 44 analyses were performed; none showed any alteration or lowering of molecular weights of the intact or constituent chains. The molecule does show unusual behavior in guanidine buffers; a shift to higher partial specific volume. However, these studies reinforce our original contention that the molecule consists of a single (A)α-chain of molecular weight 110,000 and the formulation [$(A)\alpha$, $(B)\beta_2\gamma_2$] and molecular weight 350,000±10,000. The (A)α structure is still under investigation. (Drs. J. A. Gladner, M. S. Lewis and S. I. Chung).

III. Laser Light Scattering.

The strength of cross-linked fibrin gels has been investigated by a recently developed laser light scattering technique for determining the shear modulus of soft gels. Changes in the modulus were monitored as a function of time, fibrin concentration, and concentration of Factor XIII. Rigidity measurements and SDS-polyacrylamide gels were used to correlate gel strength with the number of covalently bonded subunit chains. The modulus was found to vary linearly with the number of cross-links until maximum rigidity was achieved. Our study implies that only a faction of the crosslinks (or tie points), these occurring at points where fibers overlap, are of significance in tightening the lattice and decreasing the entropy of the network. Other crosslinking events merely cause small changes in the rigidity of individual fibers. Further chain crosslinking has little effect on the mechanical strength of the bulk sample. Thus the linkage of the points, rather than stiffening of the fibers making up the network is most important in determining clot strength. The number of these cross links for maximum mechanical strength is very small. Measurements of elasticity have been used for more than 30 years to probe the nature of fibrin gels. Such rheological methods involved direct application of a sheer force to the sample chancing that the material might be physically altered during measurement. The present method is essentially non-invasive.

Drs. R. J. Nossal and J. A. Gladner

Significance to Biomedical Research and Institute Program.

Characterization of blood coagulation factors and their mechanism of action are critical to our knowledge of cardiovascular abnormalities which may result in infarctions and strokes. Protein stabilization via crosslinking reactions by Factor XIII and similar type enzymes appear to be involved in these and other physiological processes such as wound healing, conception, etc. Studying biosynthesis and regulation of Factor XIII in organ tissue culture, uterus, will give greater knowledge of this factor in a physiological medium.

Proposed Course of Project.

Coagulation Factor XIII, and others, from cellular culture will be stressed in addition to the projects described above.

Publications:

Gladner, J.A.: Proteolytic activity produced under the influence of anti-inflammatory drugs. In Cohen, G.N. and Holzer, H. (eds). Limited Proteolysis in Microorganisms. DHEW Pub. No. (NIH)79-1591., 1979, p. 43.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 23,960-13 LBP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cell Regulation by the Action of Pharmacodynamic Agents on the Cell Membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Leonard D. Kohn, M.D. and Chief, Section on Biochemistry of Cell Regulation	Medical Director, USPHS	LBP NIAMDD LBP NIAMDD
	Evelyn F. Grollman, M.D.	Medical Officer (Research)	LBP NIAMDD
OTHERS:	George Lee, Ph.D.	Staff Fellow	LBP NIAMDD
	Francesco S. Ambesi-Impiombato, M.D.	Visiting Scientist	LBP NIAMDD
	Eduardo Consiglio, M.D.	Visiting Scientist	LBP NIAMDD
	Matityahu Fridkin, Ph.D.	Visiting Scientist	LBP NIAMDD
	Ephraim Yavin, Ph.D.	Visiting Associate	LBP NIAMDD
	Donald L. Gill, Ph.D.	Visiting Fellow	LBP NIAMDD
	Marc J. S. De Wolf, Ph.D.	Guest Worker	LBP NIAMDD
	Michael Epstein, Ph.D.	Guest Worker	LBP NIAMDD
	Ronald P. Magnusson, Ph.D.	Guest Worker	LBP NIAMDD
	Nicholas P. Morris, Ph.D.	Guest Worker	LBP NIAMDD

COOPERATING UNITS (if any)
R. M. Friedman (LEP, NIAMDD): W. H. Habig (BTB, DBP, BB, FDA): M. C. Hardegree (BTB, DBP, BB, FDA); J. E. Rall (CEB, NIAMDD); G. Salvatore (Fogarty International Center); R. J. Winand (University of Liege, Belgium)

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Biochemistry of Cell Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
10.3	9.2	1.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A structural and functional relationship in the mechanism by which glycoprotein hormones [thyrotropin (TSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), and follicle-stimulating hormone (FSH)], certain bacterial toxins (cholera and tetanus, for example), and the antiviral protective agent, interferon, transmit their message through the cell membrane has been further defined. Studies have been initiated to explore the importance of these relationships to the expression of thyroid hyperfunction in Graves' disease, to the autoimmunity of Graves' disease, to the sympathetic overactivity syndrome of tetanus, to the ability of hormones to modulate the oncogenic state, and to the mechanism by which toxins impose their pathological effects. Studies have also been initiated to evaluate the role of membranes in thyroglobulin biosynthesis and thyroglobulin biodegradation to T_3 and T_4.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The thrust of work in this section is toward an understanding of the mechanisms regulating cell activity. Special emphasis is on studies concerning the action of pharmacodynamic agents on enzymes, receptors, and lipids within the cell and in its membrane. Specifically, studies have been initiated which concern the mechanism of exophthalmos and Graves' disease; preventive therapeutic measures for these diseases; and hormone receptor function at a membrane level. These studies have been extended to the mechanisms of membrane transport in bacterial and mammalian cells; to membrane structure; to mechanisms in the biosynthesis of collagen and their relation to human collagen disease states; to the structure and function of oxidoreductases important to energetic regulation of cell functions; and to the action of toxins and interferon in subverting normal cell hormonally regulated functions to induce pathogenic states.

Major Findings:

Definition of the Structure and Function of the Thyrotropin Receptor and Relationships to Toxin Receptors. In previous work we have suggested that the thyrotropin (TSH) receptor is composed of both a glycoprotein and ganglioside component and that a critical determinant in this receptor structure is an oligosaccharide moiety. TSH upon interacting with the receptor is believed to undergo a conformational change coded for by this oligosaccharide moiety. The α subunit of TSH is presumed to enter the membrane bilayer, and the membrane undergoes a change in state or conformation. The membrane change includes an altered electrochemical gradient across the membrane; this altered gradient is believed to be an adjunct signal to the cell not mediated by cyclic AMP but obligatorily coupled or mutually regulated with cAMP changes. This structure and receptor-message link is also implication for cholera toxin. This hypothesis has been developed in the following studies:

I. (1) A 15- and 11-membered peptide of the α chain of cholera toxin which has a sequence homology to the α chain of thyrotropin and nonapeptide hormones has been synthesized. The peptides can, like cholera toxin, stimulate adenylate cyclase activity in several membrane systems. Unlike cholera toxin, but like TSH and nonapeptide hormones, the stimulation if not dependent on NAD or cytosolic factors; the peptide has no HAD hydrolase or ADP ribosylation action. Its cyclase stimulatory effect is inhibited by anticholera toxin. The data support predictions concerning functional determinants of the TSH and cholera toxin molecule derived from computer observed primary sequence analogies.

(2) Studies wherein the glycoprotein and ganglioside components of the TSH receptor are incorporated in liposomes together or separately have shown mutual interactions affecting affinity and capacity measurements. The ganglioside effect on the glycoprotein component of the TSH receptor is emphasized by similar effects on the glycoprotein component of the F_c receptor.

(3) Ramman spectroscopy has shown that gangliosides affect the ordering of the phospholipid matrix of the bilayer. There is lateral disorder but

enhanced order as measured by a decrease in transitional conformers. Cholera toxin does not induce a major disordering effect when interacting with G_{M1} in a liposome bilayer despite a major effect on bilayer permeability.

(4) The tryptophan residues of cholera toxin have been modified. With only one modified residue per β subunit, the ganglioside binding of the β subunit is completely abolished. Similarly, with only one α subunit tryptophan residue modified, the ADP ribosylation and NAD hydrolase activity of the α subunit are also abolished. The data thus indicate that the tryptophan residues are involved in both the binding site of the β subunit and the active site of the α subunit or that they are critical to the conformational integrity of the molecule. Since fluorescence studies show that these tryptophan residues are exposed, the former possibility appears most likely.

(5) Adrenergic agents stimulate the SAM-dependent conversion of phosphatidylethanolamine to phosphatidylcholine in thyroid membranes. Although the activity is exhibited by norepinephrin > epinephrin > isoproterinol, the activity is inhibited by propanolol better than phentolamine. Partial inhibition is achieved by monoamine oxidase inhibitors; no effect is exhibited by cAMP, TSH, or cholera toxin. Tetanus toxin is, however, effective in this regard, i.e., another link between tetanus and the thyroid appears evident.

(6) Using a reticulocyte system and thyroid mRNA, the glycoprotein component of the TSH receptor has been synthesized as measured by specific antibody precipitation procedures. Further characterization is being pursued.

The Tetanus Toxin Receptor Further Defined. Tetanus toxin has been shown to interact with thyroid plasma membranes. More important, the properties of this interaction closely resemble the properties of the TSH interaction with TSH receptors on these membranes; the interaction of tetanus toxin with thyroid membranes exhibit several characteristics of its interaction with neural tissue; tetanus toxin does not bind to membranes of a thyroid tumor with a TSH receptor defect; and tetanus toxin stimulates thyroid hyperfunction in mice subjected to conditions known to precipitate thyroid storm. These results predicted the tetanus receptor would be similarly structured to the TSH receptor and that tetanus toxin and TSH might have common functional elements in message transmission. This hypothesis has been extended.

(1) Tetanus toxin action has been shown to be independent of receptor expression in vitro in mice, rats, and guinea pigs, i.e., each species exhibits similar receptor properties despite > 10,000 fold differences in neurotoxic sensitivity.

(2) Retrograde transport of TSH, tetanus toxin, cholera toxin, the C fragment of tetanus toxin, and the B subunit of cholera toxin have been shown. Specificity data again emphasize the tetanus-TSH-cholera toxin receptor relationship.

(3) The binding of the B and C fragments of tetanus have been characterized using neural and thyroid membranes and ganglioside-containing liposomes. Differential lipid modulation of the binding of tetanus C fragment and TSH to

the ganglioside liposomes has been seen to be similar to differential lipid modulation of the glycoprotein component.

(4) Neuroblastoma-hybrids bind tetanus toxin. Binding is significantly poorer than to mouse or rat adult brain but is similar to fetal brain. Both the neuroblastoma-hybrid and the fetal brain binding of tetanus toxin is trypsin-sensitive; adult brain binding is not trypsin-sensitive. The possibility of a glycoprotein component to the neural tetanus receptor is thus established and is being pursued in solubilization studies.

(5) Tetanus toxin enhances Ca^{2+} efflux in synaptosomes and neuroblastoma-hybrid cell vesicles, but not in mouse Ly cell vesicles. This effect is antitoxin-inhibited and is not mimicked by toxoid. Tetanus toxin has no effect on mitochondrial Ca^{2+} efflux.

The Structure and Function of the Interferon Receptor Has Been Further Defined.

The glycoprotein fragment of the interferon receptor has been solubilized from mouse Ly and human KB-3 cells and has been reconstituted in liposomes. Tryptic fragments of this glycoprotein receptor component have been isolated from mouse Ly cell membranes. Two such fragments can enhance interferon bioactivity in vivo 10- to 100-fold. Analogous fragments from the human KB-3 cell glycoprotein receptor component are not active in this last respect. The absence of activity is correlated with a 2-fold lower binding of labeled receptor fragment to the cells by comparison to the mouse Ly fragments. Studies on the mechanism of reconstitution show the effect is: (i) specific for mouse interferon; (ii) requires preincubation of cells and receptor fragment; and (iii) is not reversible after preincubation. The glycoprotein component of the interferon receptor has been reconstituted in liposomes and its properties studied.

The Receptor Relationships Above Have Been Related to Graves' Disease.

(1) The ability of a Graves' disease gamma globulin to inhibit TSH binding has been abolished by heat treatment to 56° for 30 minutes. Inhibition has been shown to require a membrane factor released during 37° incubations of membranes. The factor is heat-stable and can be replaced by purified brain calmodulin.

(2) A tissue culture assay to measure cytotoxic antibodies in Graves' disease sera has been developed. The cytotoxic antibody recognizes the plasma membrane; cytotoxicity is complement-mediated. Cytotoxic antibodies are detected predominantly in patients with thyroiditis; however, a significant number of hypothyroid patients also have these antibodies in their sera.

(3) SV-40 transformants of cloned thyroid cells have yielded two separate lines. One has high iodide uptake and organification of the thyroglobulin. The second has low iodide uptake and no organification. Both grow rapidly. The two lines differ in their T antigen content and behave like cells in a hot and cold thyroid nodule, respectively.

A Thyroglobulin Receptor Has Been Defined.

Thyroglobulin has been shown to interact with a thyroid membrane receptor analogous to the Ashwell glycoprotein receptor on liver membranes. Receptor interaction requires preprocessing by an endogenous membrane neuraminidase. The importance of this receptor to thyroglobulin biosynthesis and degradation has been questioned. Current results show the following:

(1) Enzyme degradation studies indicate that N-acetylglucosamine is in the recognition site on the ligand.

(2) The addition binding is inhibited by mannose-6-phosphate, in analogy to lysosomal enzyme uptake processes.

(3) The receptor cross-reacts immunologically with an antisera to the Ashwell receptor on liver.

(4) Modified thyroglobulin is a potent inhibitor of TSH binding as is the endogenous thyroid neuraminidase.

(5) Sugar recognition dominates iodide recognition sites. Rubidum chloride separation procedures indicate that sialic acid content as well as iodide content can alter the resultant separations.

Significance to Biomedical Research and the Progress of the Institute:

The details of the schema outlined in the summary have implications to the pathophysiology of Graves' disease and the mechanism of action of cholera and tetanus toxin. In regard to medicinal chemistry, these relationships clearly imply caution. Yet it is hoped they will hold new opportunities to the development of peptide and carbohydrate analogs which will either block the action of cell membrane effectors which can cause a disease (cholera or tetanus toxin) or mimic the action of effectors regulating body functions (glycoprotein hormones) and blocking viral infestation of the cell (interferon). The development of agents to enhance or mimic interferon activity has been initiated; this may be important to preventing or reversing autoimmune diseases and the oncogenic state. TSH regulation of thyroid hormone production in disease states can be studied.

Proposed Course:

Studies of the TSH-EPF relationship will be continued in an attempt to examine the mechanism by which EPF activity is generated in humans in vivo. The proposed diagnostic mechanism for human exophthalmos will be further tested as will the therapeutic program. The structure of glycoprotein hormone receptors and the mechanism of action of glycoprotein hormones will be further clarified. The basis for hormone- and toxin-directed transport in mammalian cells will be further defined, and studies on thyroid cell transport will be continued. The mechanism of interferon interaction with cell membranes will be further defined as will the relationship of the glycoprotein hormones to the following bacterial toxins: cholera toxin, botulinus toxin, tetanus toxin, and various

enterotoxins produced by gram-negative bacteria. The relationships and implications to Graves' disease, exophthalmos, thyroid storm, tetanus, cholera, botulism, etc., will be pursued.

Publications:

Aloj, S. M., Lee, G., Consiglio, E., Formisano, S., Minton, A. P., and Kohn, L. D.: Dansylated thyrotropin as a probe of hormone-receptor interactions. J. Biol. Chem., in press.

Aloj, S. M., Lee, G., Grollman, E. F., Beguinot, F., Consiglio, E., and Kohn, L. D.: Role of phospholipids in the structure and function of the thyrotropin receptors. J. Biol. Chem., in press.

Chang, E. H., Grollman, E. F., Jay, F. T., Lee, G., Kohn, L. D., and Friedman, R. M.: Membrane alterations following interferon treatment. In Stinebring, W. R., and Chapple, P. J. (Eds.): Human Interferon. New York, Plenum Publishing Corp., 1978, pp. 85-99.

Consiglio, E., Salvatore, G., Rall, J. E., and Kohn, L. D.: Thyroglobulin interactions with thyroid plasma membranes: the existence of specific receptors and their potential role. J. Biol. Chem., in press.

DeWolf, M. J. S., Consiglio, E., Winand, R. J., and Kohn, L. D.: Autoimmunity, the thyrotropin receptor plus exophthalmos. Proceedings of the International Conference on Thyroid Autoimmunity, Munich, West Germany. New York, Springer-Verlag, in press.

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Grollman, E. F., Lee, G., Ramos, S., Lazo, P. S., Kaback, H. R., Friedman, R. M., and Kohn, L. D.: Relationships of the structure and function of the interferon receptor to hormone receptors and establishment of the antiviral state. Cancer Res. 38: 4172-4185, 1978.

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Hill, I. R., Levin, I. W., Lazo, P. S., and Kohn, L. D.: Effect of ganglioside and ganglioside-cholera toxin interactions on the vibrational Raman spectra of the bilayer lipid in dipalmitoyl phosphatidylcholine liposomes. Biochemistry, in press.

Kaczorowski, G., Kohn, L. D., and Kaback, H. R.: Purification and properties of D-lactate dehydrogenase from Escherichia coli ML 308-225. In Fleischer, S., and Packer, L. (Eds.): Methods in Enzymology, Biomembranes. New York, Academic Press, Vol. 53, Part D, 1978, pp. 519-527.

Kohn, L. D.: Relationships in the structure and function of cell surface receptors for glycoprotein hormones, bacterial toxins, and interferon. In Miescher, P. A., Bolis, L., Gorini, S., Lambo, T. A., Nossal, G. J. V., and Torrigiani, G. (Eds.): The Menarini Series on Immunopathology. 1st Symposium on Organ Specific Autoimmunity, Cremona, Italy, June 6-8, 1977. Basel/Stuttgart, Schwabe & Co. Ltd., 1978, pp. 137-148.

Kohn, L. D.: The thyrotropin receptor in the exophthalmos of Graves' disease. In Melnechuk, T. (Ed.): Cell Receptor Disorders. La Jolla, California, Western Behavioral Sciences Institute, 1978, pp. 28-38.

Kohn, L. D., Consiglio, E., DeWolf, M. J. S., Grollman, E. F., Ledley, F. D., Lee, G., and Morris, N. P.: Thyrotropin receptors and gangliosides. In Dreyfus, H., Mandel, P., Svennerholm, F., and Urban, D. F. (Eds.): Proceedings of the International CNRS Symposium on Structure and Function of Gangliosides, Bischenberg (Strasbourg), France, April 23-27, 1979. New York, Plenum Publishing Corp., in press.

Lee, G., Consiglio, E., Habig, W. H., Dyer, S., Hardegree, M. C., and Kohn, L. D.: Structure:function studies of receptors for thyrotropin and tetanus toxin: lipid modulation of effector binding to the glycoprotein receptor component. Biochem. Biophys. Res. Commun. 83: 313-320, 1978.

Lee, G., Grollman, E. F., Dyer, S., Beguinot, F., Kohn, L. D., Habig, W. H., and Hardegree, M. C.: Tetanus toxin and thyrotropin interactions with rat membrane preparations. J. Biol. Chem. 254: 3826-3832, 1979.

Meldolesi, M. F., Lee, G., Macchia, V., Aloj, S. M., and Kohn, L. D.: Solubilization of thyrotropin binding activity from a rat thyroid tumor with a thyrotropin receptor defect. J. Biol. Chem., in press.

Ramos, S., Grollman, E. F., Lazo, P. S., Habig, W. H., Hardegree, M. C., Kaback, H. R., and Kohn, L. D.: Effect of tetanus toxin on the accumulation of a permanent lipophilic cation ($TPMP^+$) by guinea pig synaptosomes. Proc. Natl. Acad. Sci. U.S.A., in press.

Winand, R. J., DeWolf, M. J. S., Consiglio, E., and Kohn, L. D.: Autoimmunity, the thyrotropin receptor, and exophthalmos. In Bielefeld, E. K. (Ed.): Autoimmunity in Thyroid Disease. Proceedings of the International Thyroid Symposium on Autoimmunity in Thyroid Disease, October 21-22, 1978, Munich, Germany. Stuttgart, West Germany, F. K. Shattauer Verlag, in press.

Winand, R. J., Winand-Devigne, J., Etienne-Decerf, J., Wadeleux, P. A., and Kohn, L. D.: Thyroid cytotoxic antibodies: a simple quantitative assay, their presence in thyroid diseases, and their properties. J. Clin. Endocrinol. Metab., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 23,970-04 LBP (formerly Z01 AM 31,008-03, 31,010-02, & 31,012-01 LBC)

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cancerostatic and Enzyme Regulating Agents and Intermolecular Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Koloman Laki, Ph.D. and Chief, Section on Physical Biochemistry	Research Chemist	LBP NIAMDD
OTHERS:	Sandor Damjanovich, M.D., Ph.D.	Guest Worker	LBP NIAMDD
	Judith C. Kertesz, Ph.D.	Visiting Fellow	LBP NIAMDD
	Terez Szabo, M.D.	Visiting Associate	LBP NIAMDD
	Andrew Welebir, Ph.D.	Guest Worker	LBP NIAMDD

COOPERATING UNITS (if any)

Suheil Abdulnur, Ph.D., Department of Chemistry, American University, and Sidney T. Yancey, Jr., Laboratory of Chemical Pharmacology, National Cancer Institute, and Department of Biophysics, Medical University, Debrecen, Hungary

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Physical Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(a) Neopentyl-chloroethyl-nitrosourea was found to be a cancerostatic agent in mice because of its releasing neopentyl-isocyanate, which is an active site inhibitor of transglutaminase. It is proposed that transglutaminase masks the strangeness of proliferating cells, and when the enzyme is inhibited, the cell's self defenses are eliminated by the cellular immune system. (b) E. coli RNA polymerase was assayed with different nonspecific, specific, and artificial DNAs as templates in the presence and absence of methylglyoxal. The amount of newly synthesized RNA in the presence of 10^{-8} M methylglyoxal was increased by a factor of 1.5-2.0 using calf thymus or salmon sperm DNAs or poly[d(G-C)] as templates. The poly [d(A-T)] or λ b 511 phage DNA-directed synthesis remained unaffected. The significant stimulation of the RNA synthesis was due to the increase in the number of initiated RNA chains. (c) The CNDO (complete neglect of differential overlap) method is used to investigate the binding of LiF, LiCl, NaF, and NaCl to N-methylacetamide (NMA) as a model for these ions binding to a peptide moiety. The (cation) ---O=C-N-H--- (anion) interaction is shown to result in a net residual charge on NMA, which becomes more positive as the difference in electronegativity between the anion and cation of the salt present increases. Preliminary studies indicate that salts influence the nuclear magnetic resonance spectrum of N-methyl-actamide.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

(a) *Transglutaminase and Tissue Proliferation.* In our search for compounds that inhibit transglutaminase and which are also cancerostatic, we tested several compounds. Here we report our experience with neopentyl-chloroethyl-nitrosourea (PCNU), which was synthesized at the Department of Chemistry, American University, Washington, D.C. (Drs. Laki, Szabo, and Welebir, and Mr. Yancey)

(b) *Methylglyoxal Regulating Gene Transcription.* Methylglyoxal bis-guanylhydrazone (a cancerostatic agent) is known to increase the activity of Escherichia coli RNA polymerase at low concentrations. The promotion of the transcription, however, reverted to inhibition above 3 mM concentration of the compound. The overall effect was attributed to the bis-guanylhydrazone not to methylglyoxal. The present report deals with the effect of methylglyoxal on the RNA synthesis of E. coli DNA-dependent RNA polymerase brought about by as different templates as calf thymus, salmon sperm, λ b 511 DNA, poly [d(A-T)] and poly [d(G-C)]. (Drs. Laki, Kertesz, and Damjanovich, and the Department of Biophysics, Medical University, Debrecen, Hungary)

(c) *The Interaction of Ions with N-Methylacetamide.* In a recent paper, Renugopalakrishnan and Urry, using the CNDO/2 method (CNDO = complete neglect of differential overlap), investigated the binding of Na^+ and Mg^{2+} to the carbonyl group of N-methylacetamide (NMA), as a model for these ions binding to a peptide, and found that the charges on the cations were significantly reduced, leaving a residual positive charge on NMA. This finding is of particular relevance to the proposition made in 1942 by Laki that the effect of salts on the isoelectric point of proteins may be explained if one assumed these salts to create a residual charge on the protein by interacting with the $-\overset{O}{\overset{\|}{C}}-\overset{H}{N}-$ units of the backbone. (Drs. Laki, Kertesz, and Abdulnur)

Major Findings:

(a) PCNU was dissolved in vegetable oil and diluted with saline to the desired concentration. Varying amounts of this compound was given to CDF mice at varying times after subcutaneous implantation of YLL tumor. One or 2.0 mg per mouse proved to be the effective dose. The drug given day 0 shows little effect on the survival time (14 days for the controls). Given at day 2, the drug increased survival time 30%. Given at day 7, the drug caused the tumor to disappear and doubled the survival time.

Repeated doses of 2.0 mg per mouse given weekly 4 times kept the animals tumor-free. So did a dose of 1.0 mg given twice a week. Similar effects were observed when P388, L1210, and YPC-1 tumors were implanted to the appropriate mice (syngenic conditions).

Our conclusion is that this drug is not in itself cytotoxic in the concentrations used but becomes effective when the immune system of the host becomes

geared to recognize the tumor. The drug apparently enables the cellular immune system to eliminate the malignant, proliferating cells.

We propose that transglutaminase is involved in providing the proliferating cancer cells to hide their "strangeness." By administering the nitrosourea which releases alkylisocyanate, an active-site inhibitor of transglutaminase, the proliferating cells are deprived of covering up their "strangeness."

(b) The amount of ^{14}C-AMP incorporated into the newly synthesized RNA chains with templates like salmon sperm DNA and poly[d(G-C)] showed a trend of acceleration and also inhibition at higher doses. Transcription on calf thymus DNA was enhanced only by a factor of 1.15-1.2. The same concentration range of methylglyoxal neither altered the λ b 511 phage nor the poly[d(A-T)] directed RNA synthesis in our model system.

Methylglyoxal increased the incorporation of ^{14}C-ATP even after the first minute of incubation. In order to elucidate the mechanism of these observations, kinetic measurements were carried out with (γ-^{32}P)-ATP.

The initiation of RNA chains was enhanced on the template of salmon sperm DNA. The length of the chains calculated from the bulk RNA synthesis as determined by the incorporation of ^{14}C-ATP and the number of the initiated chains remained about the same, either in the presence or in the absence of methylglyoxal. The average chain length was 16 $\pm$ 1 in both cases. However, the number of chains synthesized by one enzyme molecule was increased from 26 to 40. The calculation was carried out assuming that practically all of the enzyme molecules were in complex with DNA.

(c) The present CNDO/2 study shows that the interaction of salts with the peptide unit of NMA leads to significant net residual charges on NMA. The data show that the presence of salts affects the electronic charge densities on most of the atoms in NMA. It is interesting to note that for the four salts considered here, the largest depletion of electrons occurs in the hydrogen of -$\overset{|}{\text{N}}$-H and that the -CH_3 group attached to the carbonyl carbon acquires a net positive charge while the -CH_3 group attached to the N at the other extremity of NMA acquires a net negative charge. If similar interactions take place with the peptide backbone units in a protein, a pronounced effect on the conductivity of the protein could result.

Significance to Biomedical Research and the Progress of the Institute:

(a) This new nitrosourea as a specific inhibitor of transglutaminase may prove to be a powerful weapon to fight cancer.

(b) Our findings suggest that methylglyoxal present in animal tissues may have regulatory effects in certain kinds of processes which involve an increase of RNA synthesis. The mechanism of action is likely to be exerted through a binding to the polymerase, transcribing the DNA.

(c) Ion-peptide unit interactions are expected to become more favorable in a nonaqueous environment (such as in membranes or the interior of a protein) as well as in aqueous solutions of high salt concentration.

Proposed Course:

(a) We are engaged now in test tube experiments to see if the tumor cells after the addition of the drug became subjects for killing by sensitized T-lymphocytes.

(b) We are attempting to see in a cell-free system if RNA formed in the presence of methylglyoxal translates into some extra protein.

(c) The calculations and nuclear magnetic resonance studies are extended to see how methylglyoxal and isocyanates effect the charge distribution in N-methylacetamide.

Publications:

Laki, K., Suhai, S., and Kertesz, J. C.: Energy bands and charge transfer in proteins. Proceedings of the CIBA Foundation Symposium on Submolecular Biology and Cancer, London, England, September 24-27, 1978, in press.

Otto, P., Ladik, J., Laki, K., and Szent-Györgyi, A.: Internal charge transfer in proteins to the Schiff bases of their lysine side chains. Proc. Natl. Acad. Sci. U.S.A. 75: 3548-3550, 1978.

Tarantino, A., Thompson, P., and Laki, K.: The reaction of cancerostatic CCNU with the catalytic site of transglutaminase. Cancer Biochem. Biophys. J., in press.

Honors and Awards:

The mayor of the city of Szolnok in Hungary presented the emblem commemorating the 900th anniversary of the foundation of Szolnok to Dr. Koloman Laki, a native of that city.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,020-16 LBP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cell Surfaces: (a) Phagocytosis and Adhesion in Macrophages (b) E. coli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Loretta Leive, Ph.D.	Research Biologist	LBP NIAMDD
OTHERS:	David White, Ph.D.	Intergovernmental Personnel Act Appointee	LBP NIAMDD
	William G. Coleman, Jr., Ph.D.	Senior Staff Fellow	LBP NIAMDD
	Robert C. Goldman, Ph.D.	Staff Fellow	LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.6	3.3	1.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying the properties and structure of the outer membrane of E. coli. Mutants showing increased permeability to a large number of hydrophobic compounds include a locus in which the only apparant change is loss of lipid A phosphate, suggesting that lipid A is responsible for hydrophobic impermeability of this membrane. We have also devised a gel electrophoresis method that shows far more heterogeneity in lipopolysaccharide from a single strain than heretofore detected. In other experiments we find that the small amount of protein that copurifies with lipopolysacchride in butanol "endotoxin" preparations is all outer membrane protein, which is of interest because of the lymphocyte mitogenicity of this protein.

In studies with macrophage cell lines we have defined by gel electrophoresis several external membrane proteins that are reduced by borotritide after sialic acid oxidation, and that completely lose their label on sialidase treatment. These will be used as markers in studies of membrane dynamics. In other experiments, mutants altered in cell surface components are being isolated.

PHS-6040
(Rev. 10-76)

Project Description:

The Outer Membrane of E. coli

We previously isolated a class of E. coli mutants showing increased permeability to a large number of unrelated hydrophobic compounds and mapped it at minute 10 on the E. coli chromosome. Now we find that the sole detectable change in the organism is a loss of 90% of the phosphate in lipid A of lipopolysaccharide (LPS), and revertants losing permeability regain lipid A phosphate. These findings suggest that lipid A is the moiety responsible for hydrophobic impermeability in the outer membrane. We are now studying the hydrophobic permeability of liposomes of bacterial phospholipid, with and without normal and mutant lipid A, to test this hypothesis in an in vitro system.

Experiments in this and other laboratories have indicated that the polysaccharide of LPS of the outer membrane may be heterogeneous in length; two or three, or at most, four, size classes have been found in a given strain. Now, using sodium dodecylsulfate gel electrophoresis of very dilute solutions of LPS, we have been able to detect two dozen molecular species in an organism previously thought to have two. These represent different moieties, noncovalently joined, and are probably not multimers of a single species, as they are stable on rechromatography. This technique will permit more precise understanding of the structure of LPS.

We earlier devised a method for a rapid gentle extraction of LPS from whole cells with aqueous butanol that resulted in a product that was 90-98% LPS plus 2-10% protein. This material has received a great deal of attention from other, immunological, laboratories, as the small amount of protein that copurifies is mitogenic for mutant mice that do not respond to LPS as a mitogen. We have now shown that the proteins that copurify are specifically outer membrane proteins, which should help in the further study of the immunological phenomenon.

Macrophage Membranes and Cell Function

We have labeled membranes of macrophage cell lines by borotritide reduction of oxidized sialic acid residues, and defined several labeled protein bands on sodium dodecylsulfate gels, that completely lose their label on sialidase treatment. These proteins shall be used as markers in studies of the specificity of internalization, and rate of entry and recycling, of membrane markers during phagocytosis and pinocytosis.

Mutants of macrophage cell lines that are altered in cell surface components have been selected by isolating mutants resistant to wheat germ agglutinin, deoxyglucose inhibition, and killing by LPS. The biochemical and physiological consequences of these mutations are under study.

Publications:

Coleman, W. G., Jr., and Leive, L.: Two mutations which affect the barrier function of the Escherichia coli K-12 outer membrane. J. Bacteriol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 24,120-08 LBP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies in Traumatic Shock and Cellular Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kehl Markley, M.D. (deceased)	Medical Director, USPHS	LBP NIAMDD
OTHER:	Sanford M. Rosenthal, M.D.	Scientist Emeritus	LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.4	0.8	1.6

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

While used extensively in the clinic, the value of catecholamines and related drugs has not been established experimentally. Their effect on shock mortality was evaluated in burned mice.

Epinephrine and norepinephrine actually increased mortality when given postburn and were ineffective as pretreatment. Propanolol, a beta receptor blocking agent, decreased mortality when administered as pretreatment but had no effect post-trauma. Dibenamine lowered mortality from intraperitoneally administered Escherichia coli endotoxin.

The effect of these agents on the fall of body temperature which results from shock in mice was studied. None of these agents prevented the fall.

The effect of NaCl on longevity is a long-term study, and results will be forthcoming.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

(1) To study the function of lymphocytes and macrophages in traumatic shock in mice; (2) to elucidate the defect in cellular immunity after burn injury; (3) to study the mechanism of protective drugs in traumatic shock; and (4) to investigate the effect of NaCl in the diet of mice on extravascular fluid movement and longevity.

Methods Employed:

(1) and (2). Mice were given a moderately severe burn of 2/3 body surface area in water at 70° for 7 seconds and treated subcutaneously with 15% of body weight of 0.85% NaCl. Splenic T cells from BALB/c ($H\text{-}2^d$) mice separated on a nylon wool column were used as responding cells in the one-way mixed lymphocyte culture. Stimulating C57BL ($H\text{-}2^b$) splenic lymphocytes were irradiated with 2,000 rads. A mixture of responding and stimulating cells at a ratio of 4 : 1 was incubated at 37° with 5% CO_2 for 5-6 days. The cells were harvested, and viable cells were counted using trypan blue exclusion. Then 1-ml of sensitized cells (2×10^6) was added to 1 ml of ^{51}Cr-labeled target cells (5×10^5) and incubated for 4 hours at 37°. Cytotoxicity was measured by the amount of ^{51}Cr released from target cells into the supernatant.

(1) Migration experiments. Macrophages were collected from the peritoneal cavities of BALB/c mice. To activate macrophages, 0.3 ml Freund's complete adjuvant was injected intraperitoneally. Peritoneal macrophages were harvested 4 days later. After 3 washes, cells were suspended at a concentration of 1×10^8 cells/ml in Dulbecco's medium. Spleen lymphocytes were harvested and made up to the same concentration in Dulbecco's medium. Microcapillary tubes were 3/4 filled with the desired cell suspension (single type cell or mixture of cells), and the empty end sealed with a flame. The tubes were centrifuged and cut at the cell-fluid interface. These tubes were stuck to the side of the well of multiwell tissue culture plates (Linbro Scientific, Inc.). At least 12 wells for each migration assay were used. One ml of Dulbecco's medium was added to each well, and the plates were incubated at 37° in 5% CO_2 for 18 hours. Afterwards the patterns were fixed with glutaraldehyde. The area of migration was measured by projecting the microscopic image on a standardized bond paper, and migration fans were traced, cut out, and weighed.

To test for the production of lymphokines affecting migration, BALB/c mice were sensitized with 3×10^7 EL-4 tumor cells.

(4) Swelling of the mouse tail was measured on application of a mild vacuum to an inbred strain of mice (AIJ). Diets were prepared containing 0.5% to 4% NaCl. An apparatus for measuring blood pressure in mice is being assembled.

Major Findings:

While used extensively in the clinic, the value of catecholamines and related drugs has not been established experimentally. Their effect on shock mortality was evaluated in burned mice.

Epinephrine and norepinephrine actually increased mortality when given post-burn and were ineffective as pretreatment. Propanolol, a beta receptor blocking agent, decreased mortality when administered as pretreatment but had no effect post-trauma. Dibenamine lowered mortality from intraperitoneally administered Escherichia coli endotoxin.

The effect of these agents on the fall of body temperature which results from shock in mice was studied. None of these agents prevented the fall.

The effect of NaCl on longevity is a long-term study, and results will be forthcoming.

Proposed Course:

To determine the effect of expansion of the extracellular fluid volume on the microcirculation and longevity.

Publications:

Markley, K., and Smallman, E. T.: Effect of burn trauma in mice on the generation of cytotoxic lymphocytes. Proc. Soc. Exp. Biol. Med. 160: 468-472, 1979.

Markley, K., Smallman, E. T., and Briggs, L. W.: Effect of catecholamines and adrenergic receptor blocking drugs on early mortality and temperature regulation. J. Trauma, in press.

Rosenthal, S. M.: Physiological changes with age in the microcirculation and lymphatics. In Masuro, E. J. (Ed.): CRC Handbook of Physiological Changes with Age. Cleveland, Ohio, The Chemical Rubber Co., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 24,140-13 LBP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Chemistry and Mechanism of Pyridoxal Phosphate Enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Edith W. Miles, Ph.D. Research Chemist LBP NIAMDD

OTHER: William Higgins, Ph.D. Visiting Associate LBP NIAMDD

COOPERATING UNITS (if any)

Thomas Fairwell, Ph.D., Molecular Disease Branch, National Heart, Lung, and Blood Institute, National Institutes of Health

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of the tryptophan synthase $\alpha_2\beta_2$ complex serve as a model for studies of more complicated multienzyme complexes. Several active site residues previously identified in this laboratory have now been located in a large cyanogen bromide fragment of the β_2 subunit. The primary sequence of the first 104 residues of the β chain which contain this fragment has been determined.

Two fragments of the α subunit which result from limited tryptic proteolysis of the $\alpha_2\beta_2$ complex have been separated, characterized, and shown to recombine. The amino terminal fragment, α-1, contains residues 1-188 and has a molecular weight of 20,500; the carboxy terminal fragment, α-2, contains residues 189-268 and has a molecular weight of 8,300. The fragments are demonstrated to be independent folding domains which have no enzymatic or binding properties until recombined.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Work in our laboratory is aimed at elucidating the structure, function, and subunit interaction in the Escherichia coli tryptophan synthase $\alpha_2\beta_2$ complex. This complex is a simple model of more complicated multienzyme complexes. Specific goals are the determination of how the α and β subunits are arranged in the complex, how the active sites of the α and β subunits are arranged to form a composite active site, and how subunit interaction changes the rates and specificities of the reactions catalyzed at the active site of each subunit. One specific advantage of this system for achieving these goals is the presence of a chromophoric cofactor at the active site of the β subunit. The formation of pyridoxal phosphate substrate intermediates can be used to follow kinetics and elucidate detailed mechanisms. The change in environment of pyridoxal phosphate upon subunit association can be used as a probe of subunit association and the composite active site. Our goal of determining the chemistry of residues in the active site of each subunit is being achieved by the use of specific chemical modification reagents followed by primary sequence analysis of the modified protein chains.

Methods Employed:

Crystalline α and β_2 subunits and $\alpha_2\beta_2$ complex of tryptophan synthase are prepared and assayed by methods previously developed in this laboratory. Absorbance spectra and difference absorbance spectra are made with a Cary 118 spectrophotometer. Circular dichroism spectra and fluorescence spectra are also made. Subunit association studies are made using gel filtration. Chemical modification studies are carried out using N-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoic acid), 2-nitro-5-thiocyanobenzoic acid, iodoacetic acid, and the substrate analog L-2-amino-4-methoxy-trans-3-butenoic acid. Affinity labeling is carried out using 4-bromoacetylpyridoxamine 5'-phosphate, which has been synthesized in our laboratory. Modified enzymes are examined for their ability to combine with pyridoxal 5'-phosphate or with α subunit and for their ability to carry out partial reactions. Modified amino acid residues and peptides are identified by labeling with radioactive compounds and by techniques of proteolytic digestions, peptide mapping, ion exchange chromatography, electrophoresis, gel filtration, disc gel electrophoresis, thin-layer chromatography, nuclear magnetic resonance, mass spectrometry, infrared spectrometry, amino acid analysis, and protein sequencing. Limited tryptic hydrolysis and isolation of protein fragments are also carried out.

Major Findings:

The chemistry of the active site of the β_2 subunit of tryptophan synthase is being probed by sequence analysis of specifically modified enzymes. Three previously identified active site residues (a sulfhydryl, a histidyl residue, and a lysyl residue) have all been located in one large cyanogen bromide peptide. The total sequence of this 83-residue peptide is almost complete; location of this peptide relative to the amino terminus of the β chain results in the primary sequence of the first 104 residues of the β chain.

Our previous finding that the α subunit of tryptophan synthase can be specifically cleaved by limited tryptic proteolysis of the $\alpha_2\beta_2$ complex has been extended by separation and characterization of the two proteolytic fragments. The α-1 fragment is the amino terminal fragment (residues 1-188, molecular weight 20,500); the α-2 fragment is the carboxy terminal fragment (residues 189-268, molecular weight 8,300). The separate fragments refold into ordered structures but show no activity or ability to bind the β_2 subunit or the substrate analog indole propanol phosphate. Mixing of the two fragments restores all the properties of the α subunit. The system thus demonstrates the presence of two independently folding domains which are both essential for enzymatic activity.

Publications:

Miles, E. W.: Inactivation of tryptophan synthase by α-cyanoglycine and L-2-amino-4-methoxy-trans-3-butenoic acid. In Seiler, N., Jung, M. J., and Koch-Weser, J. (Eds.): Enzyme-Activated Irreversible Inhibitors. Proceedings of the International Symposium on Substrate-Induced Irreversible Inhibition of Enzymes, July 24 and 25, 1978, Strasbourg, France. New Amsterdam, The Netherlands, Elsevier-North Holland Biomedical Press, 1978, pp. 73-85.

Miles, E. W., and Higgins, W.: An active $\alpha'_2\beta_2$ derivative of tryptophan synthase formed by limited proteolysis. J. Biol. Chem. 253: 6266-6269, 1978.

Miles, E. W.: Tryptophan synthase: structure, function, and subunit interaction. In Meister, A. (Ed.): Advances in Enzymology and Related Areas of Molecular Biology. New York, John Wiley & Sons, 1979, Vol. 49, pp. 127-186.

Miles, E. W., and Higgins, W.: Use of limited proteolysis to probe subunit interaction in the tryptophan synthase $\alpha_2\beta_2$ complex. In Proceedings of the Fogarty International Conference on Limited Proteolysis in Microorganisms: Biological Function, Use in Protein Structural and Functional Studies, April 17-19, 1978, Bethesda, Maryland, Fogarty International Center. U.S. Department of Health, Education, and Welfare, Public Health Service Publication No. (NIH)79-1591. Washington, D.C., U.S. Government Printing Office, 1979, p. 225.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,150-08 LBP (formerly: Z01 AM 31,002-07 LBC)

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Intermolecular Interactions in Physical Biochemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Allen P. Minton, Ph.D. Research Chemist LBP NIAMDD

COOPERATING UNITS (if any)
Dr. P. D. Ross, Laboratory of Molecular Biology, NIAMDD; Drs. M. Hirshfeld and H. Flowers, Weizmann Institute of Science, Rehovot, Israel; Drs. E. Daniel and A. Klarman, Tel Aviv University, Tel Aviv, Israel

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
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INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Preliminary measurements have been made of oxygen equilibrium in dilute and concentrated solutions of normal and sickle hemoglobin. A mechanism for the desolubilization of sickle hemoglobin by nonaggregating proteins has been formulated. The binding of peanut agglutinin to synthetic carbohydrate-containing model systems is being experimentally studied. The self-association of myoglobin in concentrated solution is being experimentally studied. The effect of binding site heterogeneity upon the ligand binding equilibria and kinetics of cells and cell membranes has been theoretically studied. The exclusion of proteins from solutions containing polyethylene glycol is being theoretically studied. The effect of particle shape and size upon thermodynamic activity in heterogeneous systems is being theoretically studied.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

(1) To explore the relationship between oxygen binding and aggregation of sickle hemoglobin. (2) To characterize the concentration-dependent properties of biological molecules in terms of specific and nonspecific (excluded volume) interactions between solute molecules. (3) To explore the effect of changes in the local environment of cell surface receptors upon their ligand binding properties. (4) To explore the effect of excluded volume upon the equilibria and kinetics of biochemical reactions.

Methods Employed:

Theoretical studies employ methods of classical and statistical thermodynamics together with computer techniques for fitting theoretical model functions to experimental data. Experimental studies during the last year have utilized techniques of dual wavelength spectrophotometry, measurement of sedimentation equilibrium using the ultracentrifuge, measurement of osmotic equilibrium, and standard preparative and analytical techniques of protein chemistry.

Major Findings:

(1) Measurements have been successfully made of the oxygen equilibrium of emulsified solutions of normal and sickle hemoglobin (HbA and HbS, respectively). It has been found that above a critical hemoglobin concentration (around 15 g/dl under the experimental conditions of our experiments) the oxygen equilibrium curve of HbS diverges from those of HbA and dilute HbS, which are identical to within experimental precision. At sufficiently high concentrations of HbS the measured cooperativity of oxygen binding becomes extremely large. These observations have been rationalized in terms of models, previously formulated in this laboratory, which attribute the concentration dependence of the oxygen equilibrium of HbS to oxygen-linked aggregation of HbS. (A. P. Minton)

(2) A model for the effect of proteins which do not directly interact with HbS upon the solubility of HbS has been proposed. According to this model, which is based exclusively upon considerations of excluded volume, the size of the second protein molecule will have a large effect on the change in solubility of HbS per unit mass of second protein added. In order to explain the results obtained with hemoglobin α-chains and myoglobin, it was proposed that at sufficiently high concentrations these proteins, like hemoglobin β-chains, self-associate to form tetramers. (P. D. Ross and A. P. Minton)

(3) The self-association of cyanmet myoglobin in concentrated solutions is being investigated by means of measurements of sedimentation and osmotic equilibrium. Preliminary results from both types of experiment strongly suggest that cyanmet myoglobin does self-associate at sufficiently high concentration, probably to a tetramer. (A. Klarman, E. Daniel, M. Hirshfeld, and A. P. Minton)

(4) Model systems, consisting of glass beads to which carbohydrate groups have been covalently attached at the surface, have been prepared. The binding of

peanut agglutinin to these beads will be studied as a function of carbohydrate density, mobility, and composition. (H. Flowers and A. P. Minton)

(5) The effect of certain types of distribution of binding site affinity upon measured equilibrium binding isotherms and dissociation kinetics has been studied by means of model calculations. It has been found that some types of broad distributions of binding affinity yield isotherms which cannot be distinguished, to within conventional levels of experimental precision, from isotherms attributed to a single homogeneous class of binding sites. (A. P. Minton)

(6) Analysis of literature data on the effect of polyethylene glycol (PEG) on the solubility of proteins indicates that attractive interactions between protein and PEG (binding?) exist together with excluded volume interactions (hard core repulsion). The nature of these attractive interactions and their dependence upon protein size is under investigation. (A. P. Minton)

(7) Lattice models have been developed to permit model calculations on the effect of macromolecular size and shape upon thermodynamic activity in heterogeneous systems. Preliminary results indicate that when the volume fraction of macromolecules in a given medium is significant, the size, shape, and degree of aggregation of a macromolecule may be significantly influenced by hard-core repulsions between molecules. (A. P. Minton)

Proposed Course:

Projects (6) and (7) listed above will be continued; projects (1) and (4) will also be continued, providing the necessary collaboration can be arranged.

Significance to Biomedical Research and the Program of the Institute:

The measurements of oxygen equilibrium of sickle hemoglobin reported above provides new information about the linkage between aggregation and oxygenation in sickle hemoglobin. This information is essential to a quantitative understanding of the oxygen dependence of red cell sickling in sickle cell disease. In making these measurements a novel spectroscopic method was employed which may find more general application in the measurement of small changes in specific absorbance against a background of large changes in light scattering.

Model experiments on the binding of lectins to glass beads coated with carbohydrate "receptors" are expected to provide information as to the dependence of the binding isotherm upon the density, mobility, and composition of "receptors." This information, which at present is entirely lacking, is essential to an unambiguous interpretation of measurements of binding of hormones, drugs, and lectins to whole cells or cell membranes.

Theoretical and experimental studies of the behavior of proteins and other macromolecules in occupied solutions represent a first attempt to ascertain the differences which are likely to exist between biochemical reactions in dilute solution (typical experimental conditions in the laboratory) and the same reactions in physiological media.

Publications:

Aloj, S. M., Lee, G., Consiglio, E., Formisano, S., Minton, A. P., and Kohn, L. D.: Dansylated thyrotropin as a probe of hormone-receptor interactions. J. Biol. Chem. 254: in press.

Minton, A. P.: Analysis of oxygen equilibria in synthetic and natural valence hybrids: implications for models of heme-heme interaction in normal hemoglobin. In Caughey, W. (Ed.): Biochemical and Clinical Aspects of Hemoglobin Abnormalities. New York, Academic Press, 1978, pp. 545-557.

Minton, A. P., and Ross, P. D.: Concentration dependence of the diffusion coefficient of hemoglobin. J. Phys. Chem. 82: 1934-1938, 1978.

Pumphrey, J. G., Holroyd, K., and Minton, A. P.: Oxygen equilibrium of emulsified solutions of normal and sickle hemoglobin. Biochem. Biophys. Res. Commun., in press.

Ross, P. D., Briehl, R. W., and Minton, A. P.: Temperature dependence of nonideality in concentrated solutions of hemoglobin. Biopolymers 17: 2285-2288, 1978.

Ross, P. D., and Minton, A. P.: The effect of non-aggregating proteins upon the gelation of sickle cell hemoglobin: model calculations and data analysis. Biochem. Biophys. Res. Commun., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 24,260-13 LBP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Enzymatic Studies of Nucleic Acid Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Nancy G. Nossal, Ph.D.	Research Chemist	LBP NIAMDD
OTHERS:	Lynn L. Silver, Ph.D.	Staff Fellow	LBP NIAMDD
	Malabi Venkatesan, Ph.D.	Visiting Fellow	LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.3	3.0	0.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying the reactions required for DNA replication *in vitro* using proteins encoded by T4 bacteriophage. With single-stranded $\phi X174$ DNA as a model template, we find that ribooligonucleotide primers are made by the T4 gene 41 protein and a second protein controlled by gene 61. These primers are elongated to form duplex circular molecules by the gene 43 DNA polymerase, gene 45 protein, and the complex of gene 44 and 62 proteins (a DNA-dependent ATPase). If the gene 32 DNA binding protein is also present, the DNA is further elongated by strand displacement synthesis yielding rolling circles with long tails. The sequence of the principal primer made in the presence of the four ribonucleoside triphosphates is $pppACN_3N_4N_5$, with G and to a lesser extent C and/or U in positions 3, 4, and 5. RNA primers are initiated at many different positions on the template.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To determine the characteristics and functions of enzymes which are involved in the synthesis, modification, and degradation of DNA.

Major Findings and Proposed Course:

We are using Escherichia coli infected with T4 bacteriophage as a system in which to identify and characterize the reactions catalyzed by enzymes required for DNA replication, since this phage appears to induce the synthesis of the enzymes required for its own DNA synthesis, rather than depending on host proteins. Phage with mutations in genes coding for many of these enzymes are available due to the work of Edgar, Epstein, and their collaborators, and phage-infected bacteria can be grown in quantities allowing purification of sufficient enzyme for biochemical and physical studies. The work has been facilitated by a regulatory phage mutant characterized by Wiberg which produces high levels of several of these enzymes.

Reactions Catalyzed by the T4 Replication Proteins — Genetic studies by Edgar and Epstein et al. have shown that the proteins encoded by the phage genes 32, 41, 43, 44, 45, and 62 are required for DNA synthesis in vivo, in addition to other enzymes involved in the synthesis of DNA precursors. DNA synthesis is delayed in the absence of the gene 39, 52, 59, and 61 products. We have previously developed procedures for the purification of the gene 32 DNA binding protein; the gene 43 DNA polymerase which also has 3' to 5' exonuclease activity; the complex of the gene 44 and 62 proteins, a DNA-dependent rATP or dATPase; the gene 45 protein which stimulates the 44/62 ATPase; the gene 41 protein which has DNA-dependent nucleotidase activity; and an additional protein controlled by T4 gene 61. We showed that strand displacement DNA synthesis at nicks in duplex DNA required the gene 32, 43, 44-62, and 45 proteins and occurred in the absence of ribonucleoside triphosphates. In contrast, DNA synthesis on templates without a primer, such as the circular DNA of $\phi X174$, required in addition the gene 41 and 61 proteins and was completely dependent on ribonucleoside triphosphates.

RNA Priming of DNA Synthesis — During the past year we have shown that short ribooligonucleotides are made by the combination of the 41 and 61 proteins. This reaction requires a DNA template and is carried out on all natural single-stranded DNA's tested. Optimal RNA primer and DNA synthesis take place with all four ribonucleotide triphosphates, but there is substantial primer and DNA synthesis with only rATP and rCTP. Pentanucleotides with a 5' terminal triphosphate are the principal product of the priming reaction. Some tri-and tetranucleotide is found when the ribonucleotides are limited to rATP and rCTP. The primers made with $\gamma\ ^{32}$P-rATP have been sequenced by partial digestion with base specific ribonucleases. With rATP and rCTP, the predominant sequence is $pppACCN_4N_5$, with both A and C in position 4. With all four ribonucleotides the predominant sequence is $pppACN_3N_4N_5$, with G and to a lesser extent C and/or U in positions 3 and 4. Analysis of primers labeled at the 5' end with polynucleotide kinase, following treatment with

alkaline phosphatase, showed that there is also some G in the first position. A similar sequence distribution is found in the RNA at the 5' terminus of the DNA product.

The RNA primers are made at many different positions along the DNA molecule. Using ϕX174 DNA as a template, we find that there is alkaline phosphatase-sensitive, terminal label from γ ^{32}P-rATP in all 11 fragments formed after digestion of the product with HaeIII nuclease. Nuclease PstI makes only one cut in duplex circular ϕX174. PstI digestion of ϕX174 rolling circles with duplex tails should yield unit length linear molecules and in addition shorter fragments which are a measure of the distance between the PstI cleavage site and the end of the duplex tail. The position of the tail end is, in turn, determined by the position at which the RNA primer was made on the single-stranded template. PstI digestion of the product of the copying of ϕX174 DNA by the T4 enzymes showed more than 25 discrete minor bands which were shorter than the major unit length linear product. The T4 priming enzymes thus appear to make a family of ribooligonucleotides which are complementary to sequences found throughout any DNA molecule. Our working hypothesis is that this priming is an example of that required for the initiation of short "Okasaki" pieces on the lagging strand during the replication of duplex DNA.

Role of the Gene 61 Product — We have been trying to determine whether gene 61 is the structural gene for one of the T4 priming proteins, or encodes a protein required to activate this priming protein. The gene 61 product is made in such small quantities that it cannot be identified by comparing sodium dodecylsulfate gels of the proteins labeled after infection with T4 phage containing or lacking an intact gene 61. We have identified one minor protein band which binds to single-stranded DNA cellulose during chromatography of 61^+ but not 61^- extracts. We are continuing our efforts to purify the priming protein in order to determine whether the catalytic activity resides in this minor protein band.

Although mutations in gene 61 cause a marked reduction in DNA synthesis *in vivo*, there is some DNA synthesis especially at later times and some viable phage after infection with all known amber mutants in this gene. We have constructed phage with multiple amber mutations in gene 61 and find that they also yield some T4 DNA and phage. This observation suggests that the residual DNA synthesis is unlikely to be due to low levels of this protein produced by occasional reading through the amber mutation in the nonpermissive hosts and makes it appear more likely that another phage or host protein can to some extent substitute for the gene 61 product.

Nicking-Closing Activities — The T4 proteins described thus far initiate only on single-stranded templates. We have therefore begun a search for additional T4 nicking-closing or gyrase activities which might be required for initiation with duplex, linear T4 DNA. We have partially purified a protein (or proteins) not present in uninfected cells, which appears to catalyze the nicking-closing of supercoiled DNA.

Proposed Course:

During the next year we will try to determine the specific functions carried out by 41 and 61 proteins during the synthesis of the oligonucleotide primers. We will study the contribution of the 43, 44/62, 45, and 32 proteins to the unwinding reaction which accompanies strand displacement synthesis, since it is clear that this unwinding is not carried out by the gene 32 DNA binding protein alone.

We will search for the phage or host proteins which can apparently substitute for the priming protein controlled by gene 61 and will continue our efforts to determine if it is the structural gene for this protein.

We will continue to purify and characterize the T4 nicking-closing enzyme, especially to determine if it facilitates initiation within duplex DNA. We will also search for additional factors which may be required for origin-specific initiation of the leading strand during the replication of duplex DNA.

Publications:

Nossal, N. G.: DNA replication with bacteriophage T4 proteins: purification of the proteins encoded by T4 genes 41, 45, 44, and 62 using a complementation assay. J. Biol. Chem., in press.

Nossal, N. G., and Peterlin, B. M.: DNA replication by bacteriophage T4 proteins: the T4, 43, 32, 44-62, and 45 proteins are required for strand displacement synthesis at nicks in duplex DNA. J. Biol. Chem., in press.

Silver, L. L., and Nossal, N. G.: DNA replication by T4 bacteriophage proteins: role of the DNA-delay gene 61 in the chain initiation reaction. Cold Spring Harbor Symp. Quant. Biol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,360-05 LBP (formerly: Z01 AM 31,003-04 LBC)

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Action of Thrombin on Fibrinogen and Study of Related Blood Clotting Mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Albert J. Osbahr, Ph.D. Research Biochemist LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Biophysical Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The specific methylation of fibrinogen with dimethylsulfate resulted in modification of aspartic and glutamic acid groups which was ascertained by chromatographic analysis of the products of an enzymatic digest of the modified fibrinogen. The trimethyloxonium ion-modified fibrinogen produced an ultrastructure of the methylated fibrinogen which resembled the thrombin-induced physiological clot formation. Evidence of periodicity in the electron micrographs of the methylated fibrinogen was achieved by a low incorporation of methyl groups into the fibrinogen. High levels of modification produced a polymerization that was less ordered structurally. The proteolytic digestion of fibrinogen by plasmin is considerably altered by citraconylation, as was demonstrated by disc electrophoretic patterns. Fragments D and E usually generated from native fibrinogen were not present; however, higher molecular weight components were obtained. This is similar to observations made on patients with certain abnormal fibrinogens. Saponification of the methylated fibrinogen polymer resulted in solubilization and regenerated native fibrinogen. The action of thrombin on the demethylated fibrinogen produced a fibrin clot with the release of peptides A and B at physiological rates.

Project Description:

Objectives:

Evaluation of the structural basis for the physiological action of thrombin on fibrinogen is in progress, as well as, the biological activity of the fibrinopeptides released during the process of blood coagulation. This study involves the role of the fibrinopeptides in the mechanism of hemostasis via a mode of action resulting in vasoconstriction.

The chemical modification of functional groups is a powerful tool to probe structure-function relationships. Our particular interest centers around the amino acids with functional groups that may generate positive or negative charges in the protein structure. Two such groups are the ε-amino of lysine and the much neglected carboxyl groups of aspartic and glutamic acids. It is our hope that the chemical modification of these residues in the blood clotting proteins with specific modifying agents may significantly increase our knowledge and understanding of the mode of action of thrombin on fibrinogen, as well as, the mechanism involved in the polymerization of monomeric fibrin into the fibrin clot.

Methods Employed:

The structure and composition of peptides were analyzed by high voltage electrophoresis, liquid column chromatography, and spectrophotometric analysis. The purity of the protein and peptide systems was evaluated by ultracentrifugation, sodium dodecylsulfate gel electrophoresis, thin-layer chromatography, and paper chromatography. The determination of "active site" structure was accomplished by the use of proteolytic enzymes and chemical N-terminal and C-terminal methods.

The blood clotting proteins were modified by group-specific agents designed to alter the charge and structure of designated amino acid residues. The extent of this modification was monitored by established protein and peptide methods, such as dansylation, amino acid analysis, and specific amino acid procedures.

Major Findings and Proposed Course of Study:

Action of Thrombin on Citraconylated Fibrinogen — Bovine fibrinogen was modified with citraconic anhydride and isolated. A 1% solution of the modified fibrinogen in 0.3 M sodium phosphate buffer, pH 7.8, was reacted with 100 units of NIH thrombin and was incubated at 37° with constant stirring for 12 hours. The citraconylated fibrinogen which has a higher negative charge than native fibrinogen did not clot. This chemically-modified fibrinogen remained soluble and exhibited some properties in common with a number of abnormal fibrinogens obtained from patients with pathogenetic clotting disorders. The nature of these interactions are, at present, being investigated by sedimentation velocity experiments to determine the level of aggregation produced by the thrombic action on the modified fibrinogen, as well as the influence of the structural alteration on the polymerization stage.

Plasmin Digestion of Citraconylated Fibrinogen — The citraconylated bovine fibrinogen was digested with 0.5 units of bovine plasmin at 37° for 24 hours in 0.03 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl. The products of the digestion were analyzed by sodium dodecylsulfate disc electrophoresis and compared with a plasmin digestion of native fibrinogen. The typical fibrinogen degradation products, fragments D and E, were absent from the disc electrophoretic gel patterns.

Since electrophoresis was performed in the presence of sodium dodecylsulfate, it may be excluded that charge effects are responsible for this behavior. The plasmin-induced citraconylated fibrinogen gel patterns suggest that the molecular weight fragments produced are higher than those of fragments D and E derived from native fibrinogen. This functional alteration in the fibrinogen molecule, by modification of the lysine groups, may have reduced the number of bonds accessible to plasmin digestion. This model system may be valuable to our future understanding of the numerous abnormal fibrinogens, such as, congenital dysfibrinogenemia.

Chromatographic Analysis of Methylated Fibrinogen — Chromatographic techniques were applied for the identification of the specific amino acid residues modified in the methylation of fibrinogen. The methylated fibrinogen was digested by a sequential addition of a series of enzymes designed to fragment the molecule into small peptides and amino acids. Two-dimensional chromatography revealed the presence of the methyl derivatives of the amino acid residues. The modification of fibrinogen by diazomethane and thionylchloride resulted in the formation of β-methylaspartic acid and γ-methylglutamic acid, as well as N-methylhistidine and lysine. In addition, the O-methylated derivatives of serine, threonine, and tyrosine were represented. The application of dimethylsulfate to fibrinogen produced β-methylaspartic acid and γ-methylglutamic acid with small amounts of N-methylated histidine detectable in the chromatographic profiles. This analysis more clearly establishes the mode of action involved in the polymerization of modified fibrinogen, since the common products of each of the methylating procedures was a neutralization of negative charge in the fibrinogen molecule via a methylation of the aspartic and glutamic acid residues in the fibrinogen. These results define the mechanism of methylated fibrinogen polymerization as proceeding by way of a reduction of charge in the fibrinogen molecule. This interaction would relieve the repulsion between the fibrinogen chains, which prevented the molecule from polymerizing in its native state. The esterification process neutralized the charge of the carboxylate ion, thereby inducing polymer formation analogous to thrombic action.

Electron Microscopic Examination of Methylated Fibrinogen — The polymerized fibrinogen methylated by the highly specific trimethyloxonium ion was subjected to electron microscopic examination. This methylating agent appears to exclusively modify the carboxylic acid groups in the fibrinogen. At high levels of incorporation of methyl groups into the fibrinogen, the polymerized product was less ordered structurally, although it did resemble the network of the native fibrin clot formation. On the other hand, at low levels of methylation there was evidence of the appearance of periodicity in the electron micrographs. This is consistent with the more structurally ordered

thrombin-induced fibrin polymerization. These results suggest the participation of the carboxyl groups of the α, β, and γ chains of fibrinogen in the organization of the molecular interactions that result in the formation of periodicity during the native fibrin polymer formation.

Saponification of Methylated Fibrinogen — Bovine methylated fibrinogen was demethylated in 0.5% potassium hydroxide at room temperature for 1 hour. This process resulted in the solubilization of the modified fibrinogen. Bovine thrombin was added to the demthylated fibrinogen which resulted in the production of a fibrin clot. The fibrin polymer system was analyzed by first centrifuging the insoluble clot and the soluble supernatant. The supernatant was deproteinized and purified by separation on a Dowex 50 (X-2) column. The fibrinopeptides were isolated and identified. The data indicate that the fibrinopeptides A and B were released from the demethylated fibrinogen at physiological rates and, therefore, no denaturation of the fibrinogen had occurred during the methylation and demthylation procedures, and native fibrinogen was regenerated.

Significance to Biomedical Research and the Program of the Institute:

The understanding of thrombus formation in blood vessels is of great importance to our eventual control of undesirable hemostasis which can be quite destructive to life. Modification of the blood clotting proteins may well serve as a very informative model for the future investigation of the thrombin-induced polymerization of fibrinogen, as well as, a possible application in our ultimate control of the mechanism of clot formation in the absence of needed hemostasis.

Our studies indicate a relationship between the peptides from the blood clotting protein fibrinogen and the physiological processes in blood circulation. It is hoped that investigation of structure-function analysis of the blood clotting proteins may result in a deeper understanding of the mechanisms associated with hemostasis, vasoconstriction, and blood coagulation. The more we know concerning the action of these proteins and peptides, the easier our task could be in sorting out and defining the mechanisms related to clotting diseases.

Proposed Course:

The structure of the modified fibrinogens will be continued to more fully characterize the mechanisms involved in blood coagulation, hemostasis, and vasoconstriction. Continued efforts will be pursued to attempt to establish the involvement of carboxylate groups in the important function of periodicity in the stabilization of the clot formation. Additional studies will be initiated to show what effect the modification of fibrinogen has on the important cross-linking reactions which are necessary for the stabilization of the clot formation in the hemostatic process.

Studies will be continued to characterize the effect of plasmin fibrinogenolysis on the citraconylated fibrinogen with respect to bonds split and the specific groups involved in altering this proteolytic degradation. Emphasis

will also be given the interaction of thrombin with citraconylated fibrinogen by sedimentation velocity experiments to investigate the observed alteration of the polymerization phase.

Publications:

Nemeroff, C., Osbahr, A. J., Lipton, M., and Prange, A.: Cholecystokinin inhibits tail stimulation in rats. Science 200: 793-795, 1978.

Osbahr, A. J.: Chromatographic isolation and identification of methylated derivatives obtained from modified fibrinogen. Biochim. Biophys. Acta, in press.

Osbahr, A. J.: Fibrinogen-induced polymerization via the process of methylation. Thromb. Haemostas., in press.

Osbahr, A. J., Nemeroff, C., and Prang, A.: Centrally administered neurotensin. Eur. J. Pharmacol. 54: 299-302, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 24,390-06 LBP (formerly: Z01 AM 47,011-05 DB)

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Hormones and Receptors: Insulin and Insulin-Like Growth Factors (Somatomedins)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Matthew M. Rechler, M.D.	Medical Director, USPHS	LBP NIAMDD
OTHERS:	Alfred B. Knight, Jr., M.D.	Guest Worker	LBP NIAMDD
	Olga Z. Higa	Guest Worker	LBP NIAMDD
	Ellen E. Schilling	Guest Worker	LBP NIAMDD
	Yvonne W.-H. Yang	Guest Worker	LBP NIAMDD
	Joyce A. Romanus	Biologist	LBP NIAMDD

COOPERATING UNITS (if any) C. Grunfeld, C. R. Kahn, and G. S. King (Diabetes Branch, NIAMDD); A. C. Moses, S. P. Nissley, P. A. Short, and R. M. White (Metabolism Branch, NCI); H. J. Eisen (Developmental Pharmacology Branch, NICHHD); I. Fennoy (Neonatal and Pediatric Medicine Branch, NICHHD); A. M. Rosenberg (Univ. Manitoba)

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Biochemistry of Cell Regulation

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.3	6.0	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fibroblasts established in culture from skin biopsies have cell surface receptors for the polypeptide hormones, insulin and somatomedin. We have identified the first selective genetic defect of insulin receptors in fibroblasts cultured from a patient with leprechaunoid appearance, intrauterine growth retardation, and clinical insulin resistance. These results suggest a major role for insulin and insulin receptors in intrauterine growth.

In our studies of the insulin-like growth factor (somatomedin) multiplication stimulating activity (MSA), a polypeptide synthesized by a cloned line of rat liver cells (BRL 3A), progress has been made in the following areas: (i) characterization of a polypeptide biochemically and immunologically related to BRL-MSA and synthesized by explants of fetal rat liver; (ii) demonstration of 20- to 100-fold increased levels of MSA in fetal rat serum; (iii) characterization of growth hormone-dependent binding proteins for somatomedins in serum of rats and humans; and (iv) direct demonstration that acute metabolic effects of MSA and insulin, but not their long-term mitogenic effects, are mediated by insulin receptors.

1-6040
Rev. 10-79)

Project Description:

I. Insulin Receptors in Genetic Disorders of Insulin Resistance

Fibroblast cultures established from skin biopsies and propagated for many cell generations in vitro provide a useful cell type to study the genetic endowment of patient's cells free from short-term physiological influences. In order to study possible genetic components of resistance to polypeptide hormones such as insulin and somatomedins, we have characterized cell surface receptors for insulin and somatomedin on fibroblasts in culture. Insulin receptors on fibroblasts are normal in patients with insulin resistance and circulating antibodies to their insulin receptors (e.g., ataxia telangiectasia). By contrast, we have identified the first primary genetic defect of insulin receptors in an infant with low birth weight, facial features characteristic of leprechaunism, failure to thrive, and insulin resistance. The defect expressed in the patient's fibroblasts in culture is selective for insulin receptors and is reflected in a functional deficit of glucose uptake. These results suggest that insulin acting through insulin receptors is a major hormonal determinant of in utero growth. (M. M. Rechler, E. E. Schilling, C. Grunfeld, A. M. Rosenberg, and J. A. Romanus)

II. Multiplication Stimulating Activity

Multiplication stimulating activity (MSA) has been purified from media conditioned by a cloned line of rat liver cells in culture. Its chemical characterization is in progress. Specific competitive binding assays for MSA have been developed utilizing ^{125}I-labeled MSA binding to cell surface receptors, serum carrier proteins, and rabbit antibodies to MSA. MSA closely resembles the family of polypeptide hormones in human plasma known as somatomedins or insulin-like growth factors in its biological, chemical, and binding properties. (M. M. Rechler, S. P. Nissley, A. C. Moses, O. Z. Higa, E. E. Schilling, Y. W.-H. Yang, J. A. Romanus, A. B. Knight, Jr., P. A. Short, and R. M. White)

A possible role for MSA as an intrauterine growth factor has been suggested by two separate experimental approaches: (i) explants of term fetal rat liver studied in short-term organ culture synthesize significant quantities of MSA and (ii) serum from rat fetuses at term contain 20 to 100 times more MSA than the corresponding maternal sera. The level of MSA decreases to adult levels by 10-20 days after gestation. (M. M. Rechler, S. P. Nissley, H. J. Eisen, O. Z. Higa, A. C. Moses, P. A. Short, I. Fennoy, E. E. Schilling, R. M. White, and A. B. Knight, Jr.)

Rat and human plasma contain specific carrier proteins for somatomedins (identified by their ability to bind ^{125}I-MSA). In both rat and human serum, the concentration and molecular size of the somatomedin carrier proteins appear to be determined by the growth hormone status of the animal or patient. Hypophysectomized rats and growth hormone-deficient patients have low levels of carrier protein ($\cong$ albumin-size). Following in vivo administration of growth hormone, the concentration of carrier protein increases dramatically to normal levels, and, as in normals, the binding protein is predominantly

globulin-size. (A. C. Moses, S. P. Nissley, M. M. Rechler, and R. M. White)

Many tissues possess receptors for both insulin and MSA. Although these receptors have distinct specificities, insulin and MSA each cross-react with the receptors for the other hormone, presumably because of chemical similarities in the molecules. We have used Fab fragments prepared from antibodies to insulin receptors (obtained from the serum of a patient with a rare form of insulin-resistant diabetes mellitus) to selectively block insulin receptors (without affecting MSA receptors) and to assess the effect of this blockade on the biological effects of insulin and MSA. These studies demonstrate that both insulin and MSA stimulate glucose oxidation in isolated rat adipocytes via insulin receptors. By contrast, the stimulation by insulin and MSA of DNA synthesis in human fibroblasts in culture is not mediated by insulin receptors, but rather by receptors for MSA or other growth factors. (G. S. King, M. M. Rechler, C. R. Kahn, and S. P. Nissley)

Publications:

Anderson, W. B., Wilson, J., Rechler, M. M., and Nissley, S. P.: Effect of multiplication stimulating activity (MSA) on intracellular cyclic AMP levels and adenylate cyclase activity in chick embryo fibroblasts. Exp. Cell Res. 120: 47-53, 1979.

Bar, R. S., Levis, W. R., Rechler, M. M., Harrison, L. C., Siebert, C., Podskalny, J., Roth, J., and Muggeo, M.: Extreme insulin resistance in ataxia telangiectasia: defect in the affinity of insulin receptors. New Engl. J. Med. 298: 1164-1171, 1978.

Moses, A. C., Nissley, S. P., Passamani, J., White, R. M., and Rechler, M. M.: Further characterization of a GH-dependent somatomedin binding protein in rat serum and demonstration of a somatomedin binding protein produced by rat liver cells in culture. Endocrinology 104: 536-546, 1979.

Moses, A. C., Nissley, S. P., Rechler, M. M., Short, P. A., and Podskalny, J. M.: The purification and characterization of multiplication stimulating activity (MSA) from media conditioned by a rat liver cell line. International Symposium on Somatomedins and Growth, Santa Margherita Ligure, Italy, March 1-3, 1978, in press.

Muggeo, M., Kahn, C. R., Bar, R. S., Rechler, M. M., Flier, J. S., and Roth, J.: The underlying insulin receptor in patients with antireceptor autoantibodies: demonstration of normal binding and immunologic properties. J. Clin. Endocrinol. Metab., in press.

Nissley, S. P., Rechler, M. M., Moses, A. C., Eisen, H. J., Higa, O. Z., Short, P. A., Fennoy, I., Bruni, C. B., and White, R. M.: Evidence that multiplication stimulating activity (MSA) purified from the BRL-3A rat liver cell line is found in rat serum and fetal liver organ cultures. In Hormones and Cell Culture, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, in press.

Rechler, M. M., Schilling, E. E., King, G. S., Fraioli, F., Rosenberg, A. M., Higa, O. Z., Podskalny, J. M., Grunfeld, C., Nissley, S. P., and Kahn, C. R.: Receptors for insulin and insulin-like growth factors in disease. Neurotransmitters and Peptide Hormones, Proceedings of the 1st International Colloquium on Receptors, Capri, Italy, May 13-18, 1979. New York, Raven Press, in press.

Schilling, E. E., Rechler, M. M., Grunfeld, C., and Rosenberg, A. M.: Primary defect of insulin receptors in skin fibroblasts cultured from an infant with leprechaunism and insulin resistance. Proc. Natl. Acad. Sci. U.S.A., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,590-08 LBP (formerly: Z01 AM 31,004-07 LBC)

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structure and Interactions of Biologically Important Macromolecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Harry A. Saroff, Ph.D. Scientific Director, USPHS (retired March 1978) LBC NIAMDD

Scientist Emeritus and Guest Worker LBP NIAMDD

COOPERATING UNITS (if any)
Clinical Endocrinology Branch, NIAMDD; James C. Osborne, Ph.D., Molecular Disease Branch, NHLBI; Elvin A. Kabat, Ph.D., Columbia University College of Physicians and Surgeons, New York, New York (and Consultant to NIH at present)

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Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cooperative binding systems involving aggregation, redistribution, subunit interactions, and ligand interactions are being formulated and studied in hemoglobin and other proteins related to hormone action.

Slow degradation of hemoglobin, thyroglobulin, immunoglobulins, and albumin is under investigation in order to define and explain the phenomenon.

Monte Carlo analyses are done to yield probabilities for uniqueness and homology of amino acid sequences.

1-5040
Rev. 10-76)

Project Description:

Objectives:

The main objective of this program is an elucidation of the structure of naturally occurring macromolecules based on the methods of physical chemistry. Macromolecules are studied primarily in solution, and the emphasis is on the accumulation of data on the state of the molecule with respect to its environment. Gross features of structures as well as fine detail may be observed by studies.

Methods:

In our studies aimed at finer details of protein structure, the methods used involve the measurements of the binding of small molecules or ions to specific sites on the protein molecules. Depending on our needs, we employ ultracentrifugal, electrophoretic, light scattering, and other spectral techniques.

Major Findings and Proposed Course of Studies:

(A) Binding Studies and Protein Interactions — Model systems are being developed and studied for explaining in a reasonable manner experimental binding data. A large body of biological data appears to fall in the category of cooperative equilibria and our efforts are directed to this area. [Dr. H. A. Saroff, Clinical Endocrinology Branch (NIAMDD), and Dr. J. C. Osborne (NHLBI)]

(B) Slow (Spontaneous) Degradation of Proteins — Many examples of nonenzymatic cleavage of peptide bonds have been reported in the literature. Some of the conditions are unusually mild involving only hydrogen and hydroxyl ions. The intrinsic internal environment of protein molecules affords electronic configurations which would lend themselves to autocatalytic enzymatic action given transient high potential regions. It is reasonable to assume that the evolutionary process would, in some instances, employ this intrinsic property of the protein molecule.

In our studies on the spontaneous degradation of proteins we are investigating the precipitin reaction of immunoglobulins in a search for a covalent component of this reaction related to spontaneous degradation. Progress in these studies so far has been equivocal.

(C) Statistical Studies on Protein Sequences — In our studies on amino acid sequences of proteins we have introduced the concept of uniqueness and a method for its quantitative evaluation. We have now shown that human cytochrome c, a unique protein, has a significant probability of containing from 2 to 13 tripeptides which are capable of identifying both the region of the molecule from which the tripeptide originated as well as the cytochrome c molecule itself against the background of all the other proteins of human origin.

Our studies on protein sequences have branched out into homology evaluations on the immunoglobulins. (Drs. H. A. Saroff and E. A. Kabat)

Significance to Biomedical Research and the Program of the Institute:

Our emphasis is on the relationship between chemical properties and the structure of macromolecules. Once these properties are properly related, the attack on the relationship between structure and function can proceed on a solid basis. The proteins and macromolecules studied are either enzymes or those of interest in biological systems. Our systems are highly controlled with reference to the conditions of the protein environment and the biomedical significance of our findings derive from extrapolation to the biological system. Studies on the binding properties of protein systems related to certain hormones provide insight to possible modes of cooperative effects in hormone action.

Publication:

Saroff, H. A.: Book review of Cooperative Equilibria in Physical Biochemistry by D. Poland. Q. Rev. Biol., in press (September 1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 24,640-08 LBP (formerly: Z01 AM 31,004-07 LBC)

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Kinetics of Oxidation of Hemoglobin by Various Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard B. Simpson, Ph.D. Research Chemist LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Although it had been known that copper rapidly oxidizes the β hemes of hemoglobin, it was found that copper will also oxidize the α hemes, but at a rate of only about 20 1/M/sec at pH 6, about a thousandth as fast as for the β hemes. If the rapid oxidation of the β hemes is prevented by reacting the B-93 sulfhydryl groups with mercury or an organic thiol reagent, copper will still oxidize the β hemes, but at a rate about the same as for the α hemes.

Although the spectral changes during the reaction of hydroxylamine with hemoglobin are similar to those when ferricyanide oxidizes hemoglobin, paramagnetic resonance measurements and the relatively small decrease in absorption at 630 nm on adding cyanide to the final product, cast some doubt on whether much of the hemoglobin is oxidized by hydroxylamine.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To obtain some insight into the mechanism of reactions of hemoglobin, a study is being made of the kinetics and products of reaction with various agents that oxidize the heme.

Methods Employed:

Red cells were washed 3 times with isotonic sodium chloride and lysed with distilled water. After centrifugation, the supernatant was chromatographed in Tris buffer on DEAE-Sephadex A-50 to yield a band of hemoglobin A eluting at about pH 7.8.

The rate of hemoglobin oxidation was measured by the decrease in absorbance at 630 nm. For slow reactions the Cary spectrophotometer was used. For fast reactions the Durrum stopped-flow spectrophotometer was required. To help identify the products of reaction, the electron paramagnetic resonance was measured in the Varian apparatus operated by Dr. Hideo Kon (Laboratory of Chemical Physics, NIAMDD). The paramagnetic resonance signal distinguishes nitrosyl hemoglobin from high-spin ferric hemoglobin, the usual product of oxidation.

Major Findings:

The reaction of hydroxylamine with hemoglobin in air at pH 7.0, produces considerable nitrosyl hemoglobin, but the same reaction with deoxyhemoglobin produces much less nitrosyl hemoglobin and suggests that any nitric oxide produced from hydroxylamine is a result of oxidation by oxygen rather than by hemoglobin. The literature indicates, however, that oxidation of hydroxylamine by oxygen is very slow below pH 9.0.

In measurements of the reactivity of hydroxylamine and of ferricyanide with hemoglobin, the rate of hydroxylamine reaction showed very little pH dependence compared to that of the ferricyanide oxidation.

Although the spectral changes during the reaction of hydroxylamine with hemoglobin are similar to those when ferricyanide oxidizes hemoglobin, paramagnetic resonance measurements do not show as much high-spin ferric signal. Another test considered specific for high-spin ferric heme is the decrease in absorption at 630 nm on adding cyanide. The relatively small decrease found with the hydroxylamine-treated hemoglobin compared to that for ferricyanide oxidized hemoglobin casts some doubt on the usual assumption in the literature that the reaction of hydroxylamine produces almost entirely ferric hemoglobin.

Winterbourn and Carrell had shown for human hemoglobin, and Rifkind for horse hemoglobin that copper rapidly oxidizes the β hemes by a mechanism involving the B-93 sulfhydryl group. We find that copper also oxidizes the α hemes, but at a rate of only about 20 1/M/sec at pH 6.0. If the rapid oxidation of the β hemes is prevented by the addition of mercury or an organic thiol

reagent, copper will still oxidize the β hemes, but at a rate about the same as for the α hemes.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,710-29 LBP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Estimation, Metabolism, and Function of Amines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Herbert Tabor, M.D.	Medical Director, USPHS	LBP NIAMDD
	and Chief, Laboratory of Biochemical Pharmacology,		LBP NIAMDD
	and Chief, Section on Pharmacology		LBP NIAMDD
	Celia White Tabor, M.D.	Medical Director, USPHS	LBP NIAMDD
OTHERS:	Edmund W. Hafner, Ph.D.	Staff Fellow	LBP NIAMDD
	Murray S. Cohn, Ph.D.	Staff Fellow	LBP NIAMDD
	George D. Markham, Ph.D.	Staff Fellow	LBP NIAMDD
	Achilles A. Demetriou, M.D.	Guest Worker	LBP NIAMDD

COOPERATING UNITS (if any)

None

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Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.0	5.5	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are continuing our studies on the biological role of polyamines. Particular attention is being directed towards the selection of Escherichia coli and yeast mutants that are defective in the biosynthesis of these amines, and a study of the resultant phenotypic effects. We are also studying the control of polyamine biosynthesis in the yeast mutants.

Studies are also being carried out on the characterization of the specific enzymes involved in spermidine biosynthesis, with particular emphasis on adenosylmethionine decarboxylase from prokaryote and eukaryote sources.

We are also identifying plasmids carrying the specific E. coli genes and preparing plasmids carrying the yeast enzymes involved in the steps of polyamine biosynthesis and adenosylmethionine biosynthesis. These are being used to obtain high yields of the enzymes to permit more complete study of the proteins and cofactors involved.

Rev. 10-76)

Project Description:

Mutations in Spermidine Biosynthesis in Escherichia coli and Saccharomyces cerevisiae.

We have extended our studies of the quadruple mutants of E. coli that we have constructed which are completely deficient in polyamines and carry mutations in ornithine decarboxylase, arginine decarboxylase, agmatine ureohydrolase, and adenosylmethionine decarboxylase. These organisms have no detectable putrescine or spermidine. Their growth rate is markedly slowed; the doubling time is 5 hours. These cells, however, are able to maintain the growth of T4, T7, Mu and f2 bacteriophages. The sensitivity to some antibiotics is slightly enhanced. We are continuing studying these multiple mutants to try to define specific function for the polyamines in E. coli.

We are also isolating more mutants in adenosylmethionine decarboxylase in attempts to obtain mutations which prevent the formation of the pyruvate cofactor in the enzyme, as well as structural mutations in the enzyme.

In order to further study the pyruvate cofactor in the E. coli enzyme, we have isolated a plasmid from the Carbon collection which carries the E. coli gene for the adenosylmethionine decarboxylase. We have developed culture conditions for cells carrying this plasmid to amplify the amount of enzyme that we can obtain for study. We are developing purification procedures for large amounts of this protein.

We are isolating and mapping mutants of E. coli in lysine decarboxylase in order to eliminate the formation of cadaverine from lysine in the polyamine-deficient organism.

Studies with mutants of yeast lacking adenosylmethionine decarboxylase have yielded evidence for two specific functions of spermidine or spermine in vivo: (i) the yeast cannot sporulate and (ii) they cannot maintain the double-stranded RNA plasmid of yeast called "killer plasmid." Putrescine cannot substitute for spermidine or spermine in these functions. The gene for adenosylmethionine decarboxylase is tightly linked to the argl gene in S. cerevisiae.

We have also been isolating mutations in the ornithine decaboxylase gene to obtain yeasts that are completely deficient in putrescine as well as spermidine and spermine. One of these mutants is completely unable to grow in the absence of added spermidine or spermine. We have obtained bypass mutants of these strains and have been studying the control of the eukaryote ornithine decarboxylase. We are purifying the ornithine decarboxylase from yeast.

We have devised two rapid assays for mixed leucocyte histocompatibility; one is based on the very early striking increases in ornithine decarboxylase and adenosylmethionine decarboxylase, two of the enzymes involved in polyamine biosynthesis. We have also obtained a highly purified growth factor from regenerating liver, which acts specifically on liver cells in culture. The second is based on an unknown acetylation reaction. We are studying the mechanism of this reaction.

Publications:

Cohn, M. S., Tabor, C. W., and Tabor, H.: Mutants of Saccharomyces cerevisiae defective in the biosynthesis of polyamines from S-adenosylmethionine. In Usdin, E., Borchardt, R. T., and Creveling, C. R. (Eds.): Transmethylation. Proceedings of the Conference on Transmethylation, Bethesda, Maryland, October 16-19, 1978. New York, New York, Elsevier/North-Holland, 1979, pp. 91-93.

Cohn, M. S., Tabor, C. W., Tabor, H., and Wickner, R. B.: Spermidine or spermine requirement for killer double-stranded RNA plasmid replication in yeast. J. Biol. Chem. 253: 5225-5227, 1978.

Demetriou, A. A., Flye, M. W., Tabor, C. W., and Tabor, H.: An enzymatic method permitting early determination of histocompatibility in mixed leukocyte culture. Transplantation 27: 190-193, 1979.

Hafner, E. W., Tabor, H., and Tabor, C. W.: Mutants of Escherichia coli defective in the biosynthesis of polyamines from S-adenosylmethionine. In Usdin, E., Borchardt, R. T., and Creveling, C. R. (Eds.): Transmethylation. Proceedings of the Conference on Transmethylation, Bethesda, Maryland, October 16-19, 1978. New York, New York, Elsevier/North-Holland, 1979, pp. 85-89.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 24,940-06 LBP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Killer Double-Stranded RNA Plasmids of Saccharomyces cerevisiae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Reed B. Wickner, M.D.	Senior Surgeon, USPHS	LBP NIAMDD
OTHERS:	Akio Toh-e, Ph.D.	Visiting Associate	LBP NIAMDD
	Patricia Guerry-Kopecko, Ph.D.	Staff Fellow	LBP NIAMDD
	Kozo Ouchi, Ph.D.	Guest Worker	LBP NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Pharmacology

SECTION
Section on Pharmacology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.1	2.8	0.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The presence of a 1.4 x 10^6 dalton double-stranded RNA in intracellular virus-like particles (the K_1 killer plasmid or killer virus) in Saccharomyces cerevisiae enables cells to secrete a protein toxin and be immune to this toxin. We have defined 28 chromosomal genes (called mak1, . . .) needed to replicate or maintain the killer plasmid. We have found effects of regulatory chromosomal mutations affecting the killer plasmid (ski mutations) on the interference phenomenon by defective-interfering plasmid mutants. A second killer plasmid, called K_2, is excluded by the K_1 plasmid. We have found a new plasmid, called [EXL], which is not a killer plasmid but also excludes K_2. Yet another plasmid, called [NEX], prevents this effect of [EXL] on K_2. A chromosomal gene, man1, is needed to maintain [NEX]. Another chromosomal gene, mkt1, is required K_2 maintenance if [NEX] is present.

PHS-6040
(Rev. 10-76)

Project Description:

Background:

Some strains of the eukaryote Saccharomyces cerevisiae secrete a protein toxin which is lethal to other strains. The toxin-secreting strains are called killers and are immune to their own toxin. Two different toxin specificities are known (K_1^+ and K_2^+ phenotypes) with their corresponding immunity (R_1^+ and R_2^+) specificities. K_1 killer strains are killed by the toxin from K_2 killers and vice versa. Ability to secrete an active toxin depends on the presence of a double-stranded (ds) RNA which codes for the toxin and an immunity function. K_1 strains have a 1.25×10^6 dalton ds RNA, called M1, while K_2 killers carry a smaller 1.0×10^6 dalton ds RNA, M2. Each ds RNA is found in virus-like particles. Another ds RNA, called L (3.0×10^6 daltons), is found in virtually all S. cerevisiae strains and codes for the coat protein of the virus-like particles in which it is encapsulated. The K_1 and K_2 killer traits are inherited as plasmids (denoted [KIL-k_1] and [KIL-k_2]), and the virus-like particles containing L, M1, or M2 are not infectious.

We have previously described two chromosomal genes kex1 and kex2, needed for expressing the killer phenotype, but not needed for resistance or for killer plasmid (M1 ds RNA) maintenance. The kex2 gene is also necessary for secretion of active α hormone--a pheromone secreted by cells of α mating type which readies a cells for mating--and for meiotic spore formation. The rex1 gene is required for expression of resistance to the toxin, but not for toxin production or plasmid maintenance.

Our studies have also revealed 28 chromosomal genes needed to maintain or replicate the K_1 killer plasmid-virus (mak genes). Mutants in any of these genes result in loss of the M1 ds RNA, but not of L ds RNA. Thus, M1 and L must be replicated and maintained by very different enzymes. Several mak genes are also required for optimal growth rate, maintenance of the mitochondrial genome or meiosis.

New Findings:

MAK Genes. We have continued studying chromosomal genes needed to maintain [KIL-k_1], mak genes. Several temperature-sensitive (ts) mak mutants have been isolated, and one has been mapped on chromosome X. Two such ts mak mutants are needed for replication of one [KIL-k_1] plasmid, but not for another. Thus, killer plasmids found in various strains having the identical toxin-immunity specificity may differ significantly in their mode of replication. We have also detected a difference in the need for these mak genes in mitotic growth compared with ascospore germination.

While all mak mutants studied to date lose M1 ds RNA and retain L ds RNA, we have found that one, mak3-1, results in a 10-fold decrease in the cellular content of L ds RNA.

We have begun cloning mak, kex, and ski genes utilizing plasmid vectors able to grow in either yeast or Escherichia coli.

Defective-Interfering Plasmid-Virus. Like nearly all viruses of eukaryotes, mutants of M_1 ds RNA deleted for a substantial portion of the middle portion of the molecule interfere with replication of the normal K_1 killer plasmid but do not give the cell the K_1^+ or R_1^+ phenotypes. These mutants are called DI plasmids or suppressive plasmids ([KIL-s_1]). We have begun to study the role of the chromosomal mak and ski genes in the interference process, an area not easily approached in the animal virus systems. We have found that mutations of the ski genes adversely affect the ability of [KIL-s_1] plasmids to interfere with the [KIL-k_1] plasmids. Further, we find that mutants of [KIL-s_1] which have become replication-defective, can still interfere with the normal [KIL-k_1]. Thus, the interference is not simply a smaller genome replicating faster than a normal genome.

The [KIL-k_2] Plasmid. It has been reported that the [KIL-k_1] plasmid excludes [KIL-k_2], presumably by preventing its replication, when both are introduced into the same cell. In beginning to study this phenomenon, we have discovered another exclusion phenomenon which has led to our finding two new plasmids, called [EXL] and [NEX], and two new chromosomal genes, called mkt1 and man1. Like [KIL-k_1], the [EXL] plasmid has the ability to prevent the replication of the [KIL-k_2] plasmid when both are together in a cell. We call this property of [EXL] exclusion. [EXL] does not give cells carrying it a killer or immunity phenotype. But like [KIL-k_1], [EXL] is dependent on the chromosomal PET18 and MAK16 genes. This suggests that [EXL] may be a ds RNA plasmid, but we have not yet detected the nucleic acid corresponding to [EXL]. [EXL] does not exclude [KIL-k_1].

[KIL-k_2] strains also carrying [NEX], a plasmid distinct from [KIL-k_1], [KIL-k_2], or [EXL], are insensitive to the action of [EXL]. [NEX] is so named because it makes [KIL-k_2] nonexcludable.

The MAN1 gene is required for the maintenance of [NEX], but not for maintenance of either [KIL-k_1], [KIL-k_2], or [EXL].

The chromosomal MKT1 gene is needed for the maintenance of [KIL-k_2] in cells also carrying [NEX]. If [NEX] is absent, [KIL-k_2] can be maintained in spite of the absence of a functional MKT1 gene. This result is reminiscent of our earlier results concerning the interaction of [KIL-k_1], ρ (the mitochondrial DNA genome), and the chromosomal MAK10 gene. In that case, we found that MAK10 was needed for the maintenance of [KIL-k_1] only in the presence of ρ. If ρ was absent, MAK10 was not needed for maintenance of [KIL-k_1]. MKT1 has not yet been mapped, but is not at the MAK10 site on chromosome V.

The properties of [NEX] are somewhat paradoxical also because [NEX] helps retain [KIL-k_2] by preventing [EXL] action, but, when the cell is mkt1$^-$, [NEX] eliminates [KIL-k_2], even in the absence of [EXL].

Significance to Biomedical Research and the Program of the Institute:

Using this system, we can explore in depth in a eukaryotic cell, the relationships among different plasmids and between plasmids and host (chromosomal) genes.

Publications:

Cohn, M. S., Tabor, C. W., Tabor, H., and Wickner, R. B.: Spermidine or spermine requirement for killer double-stranded RNA plasmid replication in yeast. J. Biol. Chem. 253: 5225-5227, 1978.

Leibowitz, M. J., and Wickner, R. B.: pet18: a chromosomal gene required for cell growth and for the maintenance of mitochondrial DNA and the killer plasmid of yeast. Mol. Gen. Genet. 165: 115-121, 1978.

Toh-e, A., Guerry, P., and Wickner, R. B.: Chromosomal superkiller mutants of Saccharomyces cerevisiae. J. Bacteriol. 136: 1002-1007, 1978.

Toh-e, A., and Wickner, R. B.: A mutant killer plasmid whose replication depends on a chromosomal "superkiller" mutation. Genetics, in press.

Wickner, R. B.: Mapping of chromosomal genes of Saccharomyces cerevisiae using an improved genetic mapping method. Genetics, in press.

Wickner, R. B.: The killer double-stranded RNA plasmids of yeast. Plasmid, in press.

Honors and Awards:

In 1978 Dr. Reed B. Wickner served as a member of the National Science Foundation Advisory Committee for Biochemistry and Biophysics. He served as a member of the National Science Foundation Advisory Committee for Physiology and Cellular and Molecular Biology in 1979.

ANNUAL REPORT OF THE LABORATORY OF CHEMICAL PHYSICS,
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

Several projects have been concerned with the electronic spectra of small molecules. An understanding of these spectra is essential for the interpretation of the optical properties of much more complex biological chromophores. McDiarmid has continued her investigations of gas phase electronic spectra, with special emphasis on ethylene. The ultraviolet spectra of ethylene, deuteroethylene, and all five partially deuterated ethylenes were analyzed and assigned. McDiarmid and Doering (Johns Hopkins University) have begun an investigation of the electron impact spectrum of butadiene, and, because of the different selection rules with this method, have discovered a new electronic transition under the main $\pi \rightarrow \pi^*$ optical transition. Butadiene is the simplest molecule with many of the basic spectroscopic properties of retinal, the chromophore of the visual pigment rhodopsin. Hofrichter, Eaton, and Hochstrasser (University of Pennsylvania) have begun an investigation of the polarized single crystal spectrum of all-trans retinal, and have uncovered the forbidden "A_g" state, which is very important for understanding the photochemistry of the visual excitation process.

Charney and Lee have been investigating the physical properties of DNA, polynucleotides, and dinucleosides. They have used linear dichroism to study the mechanism of orientation of these polyelectrolytes by electric fields. By varying the electrolyte composition of the medium, they have obtained results that provide critical tests of existing polyelectrolyte theories, and that permit the development of new theories for the mechanism of orientation. Charney and Lee have also used energy minimization calculations to predict the solution conformation of dinucleosides, and have found good correspondence with those determined from nuclear magnetic resonance studies. In a similar theoretical study, Charney and Rosenfield have used the energy minimization technique to calculate the population of conformational states of cyclohexadiene and α-phellandrene. These results permitted a recalculation of circular dichroism data using the CNDO molecular orbital method, and produced much better agreement of theory and experiment.

Eaton, Hofrichter and colleagues have continued their studies on the molecular pathophysiology of sickle cell disease. Ferrone has developed a technique to study the kinetics of hemoglobin S gelation from 10^{-3} seconds to 10^{+5} seconds. The method employs a CW laser to rapidly photolyse carbonmonoxyhemoglobin S and the same laser to measure the scattered light produced by polymer formation. The observation that gelation delay times at physiological hemoglobin S concentrations and temperatures can be much shorter than capillary transit times support the kinetic hypothesis for the determination of clinical severity in sickle cell disease. Sunshine has carried out a series of experiments on inhibitors of gelation, in order to define more clearly the potential usefulness of various proposed agents. The increase in delay time produced by sodium cyanate correlated extremely well with the known clinical efficacy of this drug. This result provides an important consistency test of the criteria for therapeutically useful inhibitors, which is based on Sunshine's previous investigation of hemoglobin S in mixtures with normal adult and fetal hemoglobins.

Kon and collaborators have continued their studies on the fundamental geometric and electronic structure of metalloporphyrins and heme proteins. Kon and Inoue have used electron paramagnetic resonance and magnetic susceptibility measurements to investigate the structural factors and chemical conditions that produce dimerization of metalloporphyrins and copper(II) substituted cytochrome c and its heme peptides. They have determined well-defined structural parameters, including metal-metal intermolecular distances and skew angles for the porphyrin planes, in both copper and iron porphyrins. The study of high spin iron(III) porphyrins showed that structures other than the well known μ-oxo bridged dimer are formed.

Levin and coworkers have used Raman spectroscopy to examine conformation and molecular interactions in model membrane systems. Bush and Levin studied the temperature profiles of samples containing cholesterol or lanosterol interspersed among bilayer lipids in liposomes. Their results provide detailed information on both intramolecular and intermolecular motions and support the picture of a pocket in the unsaturated chain of α-palmitoyl-β-oleoyl phosphatidylcholine in which the steroid nucleus of cholesterol can lodge, with specific hydrogen bond formation. Meanwhile, Lavialle and Levin studied the effect of melitten (a peptide isolated from bee venom) on phase transitions in dimyristoyl phosphatidylcholine liposomes. They found evidence for a distinct "boundary lipid," containing about seven lipid molecules, directly in contact with the peptide at the lipid-peptide interface.

Craig and Levin used infrared and Raman spectroscopy to examine the trans isomer of diazene (HN=NH) and to generate a vibrational potential function for this molecule and its (hypothetical) cis analog. Their results indicate that claims in the literature for identification of the elusive cis isomer are incorrect.

NMR studies of nucleotides and polynucleotides received considerable attention during the past year. Shindo and his collaborators used ^{31}P NMR to carry out two independent investigations of the conformation of polynucleotides in nucleosome core particles. One showed that the DNA exhibits considerable flexibility, with ^{31}P relaxation data in accord with a model of isotropic motion having a broad distribution of correlation times. The second study focused on histone-free poly(dAdT)·poly(dAdT) of 145 base pairs, obtained from semi-synthetic nucleosome particles. Two well defined ^{31}P lines were found, which are attributed to an alternating conformation of the phosphate backbone.

The measurement of NMR spin-lattice relaxation times (T_1) provides much useful information, of the sort described in the preceding paragraph. However, experimental determinations of such data often require large blocks of scarce spectrometer time. Becker and his collaborators have investigated the design of T_1 experiments and have suggested ways of optimizing the choice of method and experimental parameters to obtain maximum precision in the minimum experimental time. In addition, they proposed new methods that alleviate certain systematic errors present in some circumstances with conventional techniques.

Ziffer and Imuta continued their development of methods for carrying out asymmetric reductions of ketones in order to obtain alcohols of known absolute

stereochemistry and high optical purity. In order to circumvent problems arising with conjugated ketones, they have examined the enantioselective hydrolyses of a series of acetates of racemic alcohols using a strain of *Rhizopus nigricans*. The alcohols formed in these hydrolyses were optically active, with enantiomeric excesses of 50-70 per cent. A relation of predictive value between the favored product and the relative sizes of substituents on the carbinol carbon was suggested.

Weiss and Harmon have extended their study of azulene pigments in the latex of certain mushrooms to a well known edible species, *L. deliciosus*. They have discovered a previously unreported constituent, which has now been identified as a linoleate ester--a very uncommon type of compound in nature. In addition, they have completed the structure elucidation of the alkaloid rohitukine, extracted from the Indian drug plant *Amoora rohituka*.

Robinson, George and Hagins have carried out further metabolic studies on the nucleoside phosphates of retinal rod outer segments. Analyses of the adenine and guanine nucleotides by chemical and isotopic tracer methods have shown that light causes large fast changes in the cytoplasmic nucleotide levels that may be an important link in visual excitation. Yoshikami and Hagins have developed new methods for assessing the role of calcium ions in visual excitation. It has been shown that calcium ions control the dark current of retinal rod cells by a faster and more direct mechanism than does visible light.

Sharpless, Adams and Jennings have continued computerized molecular modeling on a variety of drugs and visual pigment chromophores. The value of such studies in determining three-dimensional molecular structures is still to be established, but if the method is successful, it will be a valuable addition to X-ray crystallography and magnetic resonance.

Adaptation to a single environmental stress such as cold, exercise or hypoxia has been thought to be useful in increasing tolerance to a totally different stress. Rattner and Altland have studied cross-adaptive effects induced by long term exposure to cold, hypoxia or physical training on hypobaric decompression in mice. Predive adaptation to cold or hypoxia had either no effect and actually reduced the tolerance to decompression sickness. However, physical training dramatically reduced the incidence of bends. Possible mechanisms advanced to explain this finding include: reduction of nitrogen uptake due to lean body conformation, increased capillarization of muscles to increase the transfer of gases, and improved cardiac function to improve nitrogen elimination.

Jennings, together with the staff of the Computer Systems Laboratory, DCRT, has begun the conversion of the laboratory data logging and processing system from a centralized computer to a network of microprocessors located at individual laboratory instruments. Two instruments, a spectrophotometer and an electron spin resonance spectrometer have been fitted with microprocessors to date. The new network is scheduled for completion in early 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29000-07-LCP

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Nuclear magnetic resonance: New methods and molecular structure determination

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edwin D. Becker	Research Chemist	A LCP
OTHER:	Rolf G. Tschudin	Electronic Engineer	A LCP
	Cherie L. Fisk	Staff Fellow	A LCP
	Heisaburo Shindo	Visiting Scientist	A LCP

COOPERATING UNITS (if any)

H. T. Miles (NIAMDD-LMB)
J. A. Ferretti, G. Weiss (DCRT)
R. K. Gupta, Institute for Cancer Research
T. J. Pinnavaia, Michigan St. Univ.
R. T. Simpson (NIAMDD-LNE)
J. D. McGhee (NIAMDD-LMB)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

NMR studies of nucleotide conformation and nucleotide interactions were partly directed toward the elucidation of the molecular structures of the self-associated form of guanosine-5'-phosphate at neutral pH. By direct measurement of one of the equilibria by proton NMR we found that the association leads to an octamer, the detailed structure of which has been deduced from molecular models and other NMR measurements. Another major area of interest was the study of polynucleotides in nucleosome core particles. In poly(dAdT)·poly(dAdT) of 145 base pairs ^{31}P NMR studies showed that the phosphate backbone probably has an alternating conformation.

Strategies for measuring NMR spin-lattice relaxation times have been explored from the standpoint of minimizing random errors and thus decreasing the instrumental time required to make such measurements. Modified methods have been proposed to alleviate certain systematic errors found in conventional procedures.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1. Elucidation of the forces within and between molecules, especially those of potential biological importance.

2. Development of spectroscopic methods for studying molecular structure and analyzing materials of chemical and biological interest.

Major Findings and Future Plans:

Further NMR studies of the self-association of the sodium salt of guanosine-5'-phosphate at neutral pH have been undertaken to elucidate the nature of the complexes formed. In addition to verifying some of the ^{13}C relaxation data described in last year's report, we have used proton NMR to measure directly the equilibrium constant for formation of a species giving two well defined NMR lines for H-8. This species was believed to be a head-to-tail octamer on the basis of the H-8 chemical shifts. The new results firmly establish the 8'th order concentration dependence of the equilibrium and confirm the previous assignment. Further studies are aimed at elucidation of the structures of other species present. (Fisk, Raiford, Becker)

Two independent studies by ^{31}P NMR of the conformation of polynucleotides in nucleosome core particles have been completed. The first, in collaboration with McGhee and Cohen, showed that the DNA (145 $\pm$ 3 base pairs) in nucleosome core particles exhibits considerable flexibility, with the ^{31}P relaxation data in accord with a model of isotropic motion having a broad distribution of correlation times. The ^{31}P linewidth was large, especially at high observation frequencies--a fact attributed to the variation of ^{31}P chemical shift with different base environments along the chains. This explanation was confirmed in a second study, in collaboration with Simpson, which focused on histone-free poly(dAdT)·poly(dAdT) of 145 base pairs, obtained from semi-synthetic nucleosome particles. Two well defined ^{31}P lines were found, which are attributed to an alternating conformation of the phosphate backbone. (Shindo)

The measurement of NMR relaxation times provides much useful information on molecular structure and molecular motion. However, experimental determination of such data, especially T_1, the spin-lattice relaxation time, is often a time-consuming experiment. In collaboration with Weiss, Ferretti and Gupta, we have been exploring means to minimize the random errors associated with such studies, as well as investigating novel approaches to such measurements. (An improvement of, say, 20% in experimental precision is equivalent to a time-saving of 40%--a significant factor in increasing the productivity of expensive, heavily used spectrometers.) We have shown that under the conditions of "ideal pulses" the fast inversion-recovery method is the most efficient of the commonly used methods, and we have recently suggested a modification of this method to minimize the effect of certain systematic errors. We also proposed a somewhat different approach to T_1 measurement, the "variable perturbation method," which is advantageous in measurements with certain transient molecules. Further studies are now aimed at generalizing these results to situations where instrumental limitations preclude use of the "ideal pulse" approximation. (Becker)

Publications:

Becker, E. D., Ferretti, J. A. and Gambhir, P. N.: Selection of optimum parameters for pulse Fourier transform NMR. Anal. Chem. In press.

Weiss, G. H., Gupta, R. K., Ferretti, J. A. and Becker, E. D.: The choice of optimal parameters for measurement of spin lattice relaxation times. I. Mathematical formulation. J. Mag. Res. In press.

Becker, E. D., Ferretti, J. A., Gupta, R. K. and Weiss, G. H.: The choice of optimal parameters for measurement of spin-lattice relaxation times. II. Comparison of saturation-recovery, inversion-recovery and fast inversion recovery experiments. J. Mag. Res. In press.

Gupta, R. K., Weiss, G. H., Ferretti, J. A. and Becker, E. D.: A variable perturbation method for nuclear spin-lattice relaxation measurements. J. Mag. Res. In press.

Raiford, D. S., Fisk, C. L. and Becker, E. D.: Calibration of methanol and ethylene glycol nuclear magnetic resonance thermometers. Anal. Chem. In press.

Niu, C-H., Bertrand, R. D., Shindo, H. and Cohen, J. S.: Cross-peptide bond ^{13}C-^{15}N coupling constants by ^{13}C and J cross polarization ^{15}N NMR. J. Biochem. Biophys. Methods. In press.

Shindo, H., Simpson, R. T. and Cohen, J. S.: An alternating conformation characterizes the phosphodiester backbone of poly(dAdT) in solution. J. Biol. Chem. In press.

REPORTED IN PRESS - 1978

Becker, E. D.: Nuclear magnetic resonance: recent developments in techniques and their potential application of forensic research. In Klein, M., Kruegel, A. V. and Sobol, S. P. (Eds.): Instrumental Applications in Forensic Drug Chemistry. Washington, U.S. Government Printing Office, 1979, pp. 70-79.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29001-07-LCP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Molecular dynamics and vibrational characteristics of membrane assemblies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ira W. Levin	Research Chemist	A LCP
OTHER:	Francoise Lavialle	Visiting Fellow	A LCP
	Norman C. Craig	Guest Worker	A LCP
	S. Fowler Bush	Guest Worker	A LCP

COOPERATING UNITS (if any)

D. Engelman, Yale University
C. Huang, University of Virginia
L. Kohn, LBP-NIAMDD
I. R. Hill, Southampton University
C. Mollay, Austrian Academy of Sciences

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Vibrational Raman and infrared spectroscopic transitions are used to probe the dynamical and structural properties associated with lipid-lipid and lipid-protein interactions in both real and model membrane-bilayer systems. As an example, temperature profiles of multilamellar lipid assemblies composed of either saturated or mixed saturated and unsaturated hydrocarbon chains were compared to profiles for systems containing cholesterol or lanosterol intercalated within the bilayers. Examination of the profiles relevant to interchain interactions and, separately, to intramolecular processes provides detailed information concerning the function and packing characteristics of sterols in membranes.

The properties of intrinsic and extrinsic membrane components were examined by Raman spectroscopy. The effects of melitten (a peptide isolated from bee venom) and its fragments on the thermotropic behavior of 1,2-dimyristoyl phosphatidylcholine liposomes were studied as a function of protein concentration. The existence of a distinct boundary lipid, composed of about seven lipid molecules surrounding the peptide, was established for this lipid-protein interface.

Project Description

Within the project area of the vibrational dynamics of membrane components and membrane assemblies our objectives are to probe, clarify and interpret the vibrational spectroscopic characteristics of biological membranes and related systems. The general approaches include: (a) analyses of the Raman and infrared spectra of selected systems, and (b) the determination of reliable intramolecular force fields when applicable.

A variety of laser Raman and infrared spectroscopic procedures provide the required data for the various vibrational analyses. The intramolecular force field computations are necessarily performed on a large digital computer.

The studies during the past year were partitioned into roughly two separate areas; first the clarification of specific membrane bilayer processes as reflected by the vibrational spectra of model and real membrane assemblies; and second, the continued development of techniques for reliably determining potential functions for moderate size molecules. These investigations are described in the following paragraphs.

(1) Although a potential phospholipid role for the function or expression of ganglioside and glycoprotein receptor components for interactions involving toxins and hormones has been suggested, the extent of the structural reordering occurring within the phospholipid bilayer as a result of an effector-receptor interaction remains unknown. We have applied Raman spectroscopy toward investigating the behavior of dipalmitoyl phosphatidylcholine (DPPC)-water liposomes perturbed first by the intercalation of the G_{M1} ganglioside within the lipid matrix and then the changes which arise on exposure of the model receptor system, thus created, to cholera toxin.

In particular, vibrational Raman spectra of 1,2-dipalmitoyl phosphatidylcholine (DPPC) multilayers containing 15 mole % G_{M1} were recorded as a function of temperature for the 1000-1170 cm^{-1} and 2800-3000 cm^{-1} carbon-carbon (C-C) and carbon-hydrogen (C-H) stretching mode regions, respectively. Temperature profiles were constructed using the peak height intensity ratios I_{2880}/I_{2940} and I_{1130}/I_{1100} to reflect changes in the lattice intermolecular and hydrocarbon chain intramolecular disorder parameters. Both spectroscopic markers indicated an increase of 2.6-3°C in the gel to liquid crystalline phase transition temperature and a broadening of the phase transition interval of 4.5-7.5°C. Further, the data demonstrated that G_{M1} enhances the lateral or intermolecular disorder of the DPPC liposomes throughout the gel state, while concomitantly increasing the intramolecular order of the lipid acyl chains. Cholera toxin was added in small concentrations ($\sim 4 \times 10^{-6}$ M solutions of toxin) to the G_{M1} containing DPPC liposomes at 18°C. Analysis of the resulting 1100 cm^{-1} C-C stretching mode region indicates the existence of two classes of lipid molecules; namely, a small concentration of disordered lipid molecules, characteristic of DPPC multilayers in the 39.4-44°C phase transition range, clustered within the ordered low temperature DPPC bilayer

matrix. We suggest that the disordered lipids are clustered primarily about the ganglioside components. (Hill, Kohn and Levin)

(2) Vibrational Raman and infrared spectroscopic techniques were applied toward describing the packing characteristics and associations of lipids and sterols in membrane bilayers.

(a) Extensive Raman spectroscopic temperature profiles were determined for cholesterol and lanosterol interspersed among the bilayer lipids for liposomes prepared from either dipalmitoyl phosphatidylcholine (DPPC) bilayers or α-palmitoyl-β-oleoyl phosphatidylcholine (POPC) bilayers. In comparison to the POPC system, DPPC multilayers represent a relatively tightly packed rigid gel-phase lattice as a consequence of the saturated acyl chains. POPC mimics a natural system in that the β-chain is unsaturated with the double bond at the 9,10 position. Our thesis is that the oleoyl chain forms a pocket for the steroid nucleus of cholesterol and that the 3β-OH group of cholesterol forms a hydrogen bond to the carbonyl of an α-chain of the neighboring lipid. Lanosterol is chosen for comparison with cholesterol as the additional methyl groups on both faces of the steroid nucleus are expected to inhibit the van der Waals interactions participating in the hydrophobic bonding schemes within the lipid lattice. The melting curves reflecting the chain intermolecular and, separately, the intramolecular processes for the hydrocarbon region of the bilayer supports the model with respect to the suggested lipid-sterol associations. In particular, the broadening of the gel to liquid crystalline phase transitions, an increase in the POPC-cholesterol phase transition temperature (T_c) relative to that for the POPC-lanosterol system, and the relative disordering characteristics of cholesterol and lanosterol enable a detailed model to be presented for the structure of the prepared bilayer in terms of the membrane's dynamic properties. (Bush, Huang, Levin)

(b) The conformational inequivalence of the α- and β-chains in dipalmitoyl phosphatidylcholine (DPPC) bilayers is reflected by the appearance of two carbonyl modes in both the infrared and Raman spectra of pure DPPC and DPPC-cholesterol systems. A comparison with α-palmitoyl phosphatidylcholine, which contains only one acyl chain, identifies the higher vibrational component with the α-chain of DPPC. Observation of the splitting patterns in the carbonyl region provides detailed information on (i) overall chain rotation (as distinct from the trans-gauche isomerization processes), (ii) changes in the populations of various acyl chain conformers determined by angle rearrangements in the interface region of the lipid, (iii) hydrogen bonding effects between cholesterol and particular conformations of the α-chain, and (iv) the effect of the β-face of cholesterol on the dynamical and structural properties of the β-chain of the phospholipid. (Bush and Levin)

(3) In assessing lipid-protein interactions, it is convenient to consider two classes of associations; namely, the hydrophobic interactions which immobilize the lipid chains and the polar interaction between the lipid headgroups and the charged regions of the protein. The latter

occurs at the aqueous interface. Melitten, a low molecular weight polypeptide, isolated from bee venom, provides a particularly apt system for partitioning and clearly defining the various effects arising in protein-membrane associations.

(a) Within the lipid matrix the interface between the hydrophobic region of the protein and the lipid chains has been postulated to be the primary region of lipid-protein interaction. The lipids in direct contact would presumably form an annulus or boundary lipid whose existence and properties have not been well defined or characterized. Although NMR, ESR and calorimetric studies suggest the existence of a boundary lipid, the evidence to support this concept is still quite limited and circumstantial. In applying Raman spectroscopy to this problem, we investigated the spectroscopic behavior of dimyristoyl phosphatidylcholine (DMPC) liposomes containing various concentrations of melitten. Pure DMPC liposomes exhibit a gel to liquid crystalline phase transition at 23°C. Using the C-C stretching region of the DMPC lipid chains as a probe, Raman spectroscopic temperature profiles show a decrease in the phase transition temperature T_c to 11° and 16.5°C for multilayers containing 10:1 and 14:1 lipid:protein ratios, respectively. This effect is expected for a system, such as melitten, which is capable of both electrostatic binding at the membrane surface and hydrophobic interactions within the membrane. Both the 10:1 and 14:1 lipid-protein systems show a second, relatively broad transition centered around 30°C which we attribute to the melting behavior of the immobilized boundary lipid. The 10 and 14:1 lipid-protein systems both indicate that about 5-7 lipids are implicated in the lipid-peptide complex. Temperature profiles based upon the acyl chain C-H stretching region of the Raman spectra are in accord with these conclusions. (Lavialle and Levin)

(b) In order to characterize more completely the effects of intrinsic and peripheral bilayer components on membrane functions, the effect on membrane behavior of melitten, its apolar fragment F_1 (residues 1-19) and its polar, basic fragment F_2 (residues 20-26) were-examined in DMPC liposomes with a lipid to protein mole fraction ratio of 25:1. For temperature profiles reflecting the intramolecular acyl chain trans-gauche isomerization, the hydrophobic fragment F_1 depressed the DMPC transition temperature T_c by about 4-5°C and broadened the pure DMPC transition range from ∿0.5° to ∿8°C. The polar fragment F_2, which is electrostatically attached to the bilayer headgroup region increases T_c by about 1°C, but does not significantly broaden the phase transition. Complete melitten (residues 1-26) broadens the transition and slightly depresses T_c. The peripheral component F_2 immobilizes ∿6 lipid molecules over a broader temperature range than F_1. The board melting range for the boundary lipid in whole melitten reflects the importance of the polar fragment.

The C-H stretching region spectra of DMPC plus melitten and its fragments do not reflect the presence of the boundary lipid in the 25:1 lipid-protein liposomes, although 10 and 14:1 lipid-protein ratios show the phenomenon. Melitten disorders the gel phase of the DMPC liposomes as a consequence of

the hydrophobic region of the protein and orders the liquid crystalline phase, also a consequence of the hydrophobic moiety. The polar fragment contributes little to intermolecular disordering although F_2 does introduce some additional gauche structures within the lipid acyl chains. (Lavialle, Mollay, Levin)

(4) The cis and trans isomers of diazene (HN=NH) provide the parent system for a wide variety of azo compounds. These molecules, as well as several structurally related species, have been the focus of recent studies designed to clarify the chemical and spectroscopic effects of disposing substituent groups about the nitrogen-nitrogen double bond. In view of the lack of a consistent vibrational assignment for trans-diazene, we developed a complete assignment for the fundamental modes for the trans species and its $-d_1$ and $-d_2$ isotopic modifications. A normal coordinate analysis was determined for the trans-isomer using a 7 parameter internal valence force field. A potential function for cis-diazene was generated from potential functions used to describe trans-methyldiazene, trans-diazene and cis-methyldiazene. From our calculated vibrational frequencies for cis-diazene and its deuterium isotopic components, we conclude that the claims in the literature of having identified this particularly elusive substance are incorrect. (Craig and Levin)

(5) Unfiltered argon-ion laser radiation contains numerous plasma lines which are potentially useful for calibrating spectrometers, especially when an internal standard is desired. Surprisingly, the one extensive table of plasma lines for the argon-ion laser that appears in the literature has both errors in assigning the plasma transitions correctly and a limited wavelength accuracy. Consequently, the existing list of lines is inadequate for most spectroscopic purposes. We have prepared a reliable table of plasma lines suitable for identifying plasma transitions and for calibration purposes. The listing of transitions includes relative intensities, wavelengths in standard air, wavenumbers in vacuo and wavenumber shifts relative to the two principal laser lines. (Craig and Levin)

Significance to Biomedical Research and the Program of the Institute

Raman spectroscopy offers a potentially valuable approach toward examining dynamical properties of natural and model membrane bilayer assemblies since no membrane perturbing agent is introduced by the technique. This is particularly important for investigating conformation changes induced in bilayer systems either by changes in temperature or by addition of membrane substituents and for clarifying lipid-lipid and lipid-protein interactions within the bilayer.

Proposed Course of Project

(1) Studies on the effects of anesthetics on the thermotropic behavior of model membranes will continue. Interest will be particularly focused on the ability of halothane ($CF_3CHBrCl$) to alter the size of the cooperative unit of bilayer lipids undergoing order-disorder phase transitions.

(2) Further characterization of the properties of boundary lipids surrounding proteins will continue using deuterated lipid species as markers.

(3) Studies will continue on (i) the packing characteristics of cholesterol-phospholipid complexes in bilayers and (ii) the differentiation between in-side and out-side bilayer surfaces by Raman techniques.

(4) Resonance Raman studies on selected proteins in the ultraviolet spectral regions will be emphasized.

Publications

Hill, I. R. and Levin, I. W.: Vibrational spectra and carbon-hydrogen stretching mode assignments for a series of n-alkyl carboxylic acids. J. Chem. Phys. 70: 842-851 (1979).

Hill, I. R., Levin, I. W., Lazo, P. S. and Kohn, L. D.: Effect of ganglioside and ganglioside-cholera toxin interactions on the vibrational Raman spectra on the bilayer lipid in dipalmitoyl phosphatidylcholine liposomes. Biochemistry. In press.

Craig, N. C. and Levin, I. W.: Vibrational assignment and potential function for trans-diazene (diimide); predictions for cis-diazene. J. Chem. Phys. In press.

Craig, N. C. and Levin, I. W.: Calibrating Raman spectrometers with plasma lines from the argon-ion laser. Applied Spectroscopy. In press.

REPORTED IN PRESS - 1978

Pearce, R. A. R. and Levin, I. W.: Normal coordinate treatment for propane: force constant selection through a backward elimination regression-refinement procedure. J. Chem. Phys 70: 370-379 (1979).

Levin, I. W. and Pearce, R. A. R.: Absolute infrared intensities of cyclopropane-d_0 and cyclopropane-d_6 dipole-moment derivatives and polar tensors. J. Chem. Phys. 69: 2196-2208 (1978).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29002-06-LCP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Chemistry and biosynthesis of natural compounds, and instrumental methods used in their study

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ulrich Weiss	Research Chemist (Scientist Emeritis)	A LCP
	Alan D. Harmon	Staff Fellow	A LCP

COOPERATING UNITS (if any)
James M. Cook, Department of Chemistry, University of Wisconsin, Milwaukee
J. Michael Edwards, School of Pharmacy, University of Connecticut, Storrs
Surendra P. Bhatnagar and K. M. Madyastha, India

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structure of rohitukine, an alkaloid from the Indian drug plant Amoora rohituka (synonym Aphanamixis polystachya) has been established, mainly by X-ray crystallography. It is 8-(cis-3'-hydroxy-N-methyl-4'-piperidyl)-2-methyl-5,7-dihydroxychromone. The compound is the first alkaloid from a plant of the family Meliaceae to have been structurally elucidated. At least five other bases closely related to rohitukine have been found and are being studied. The orange-colored latex of the edible mushroom Lactarius deliciosus contains the linoleate ester of a hydroxylated dihydroazulene, in addition to the parent alcohol and its stearate, which had been characterized elsewhere a number of years ago. Linoleate esters are rare in nature.

PHS-6040
(Rev. 10-76)

Major Findings, Future Plans

1. In continued cooperation with Dr. Kenneth L. Kirk, LC-NIAMDD, the preparation and pharmacological action of 2-fluoromorphine are to be studied in greater detail. The entire series of 1- and 2-halogenomorphines and -codeines is to be prepared and examined.

2. The study of novel cyclopentanoid ring-systems accessible through reaction of dimethyl 3-ketoglutarate with 1,2-dicarbonyl compounds is being continued in cooperation with Prof. J. M. Cook and his students. The tetraketo derivative of staurane (see last year's report) is being converted to the parent hydrocarbon, staurane, itself, and to the derived tetraene. Preparation of other unusual cyclopentanoid hydrocarbons is under way, and the reaction of ketoglutarate with suitable 1,2-dicarbonyl compounds is to be utilized for the synthesis of certain novel naturally-occurring sesquiterpenes. cis-Bicyclo[3.3.0]octane-3,7-dione, a compound which has become very readily accessible through work in the earliest stages of this research project, has been utilized in two outside laboratories (Universities of Pennsylvania and Nagoya, Japan) for essentially identical syntheses of an analog of the important hormone prostacyclin. The compound so obtained shows promising activity in preventing clotting of blood platelets.

3. Azulene pigments from the latex of mushrooms of the genus Lactarius. Dr. Harmon has extended his study to the well-known edible species L. deliciosus. Earlier work in Prague, Czechoslovakia had shown that the bright orange color of the latex of this species is caused by two extremely unstable dihydroazulenes, a free primary alcohol and its stearate ester. Specimens collected in Wyoming have now yielded those two pigments and an additional minor one which is the linoleate ester of the primary alcohol. This pigment may have been present in the earlier work by the Czech authors but may have escaped detection. Linoleate esters are quite uncommon in nature.

4. Alkaloids from Amoora rohituka (Meliaceae). Besides the alkaloid mentioned in last year's report--which we have named rohitukine--, extracts of this drug plant from India have yielded 5 other alkaloids, 3 of them in pure, crystalline form. Their chemistry is being studied. Like rohitukine, they are all derived from 2-methyl-5,7-dihydroxychromone (nor-eugenin), differing from it only in the structure of the nitrogen-containing moieties attached at position 8. Noreugenin was also isolated from the crude extract.

5. Work on the synthesis and biosynthesis of morphine and/or its derivatives, initiated when Dr. S. P. Bhatnagar was at NIH, is to be resumed in cooperation with him and Dr. Madyastha (Bangalore).

Publications

Harmon, A. D., Weiss, U. and Silverton, J. V.: The structure of rohitukine, the main alkaloid of Amoora rohituka (Syn. Aphanamixis polystachya), Meliaceae. Tetrahedron Letters 721-724, (1979).

Weiss, U., Fales, H. M. and Weisgraber, K. H.: Pigments of Elsinoe Species. Part VII. Revised structure of a synthetic intermediate; formation of a dibenzo-2,2'-oxidodiphenoquinone. Justus Liebig's Annalen der Chemie, in press.

Bazan, A. C., Edwards, J. M. and Weiss, U.: Synthesis of lachnanthocarpone [9-phenyl-2,6-dihydroxyphenalene-1(6)-one] by intramolecular Diels-Alder cyclization of a 1,7-diaryl-heptanoid orthoquinone; Possible biosynthetic significance of Diels-Alder reactions. Tetrahedron 34, 3005-3015 (1978).

Mitschka, R., Cook, J. M. and Weiss, U.: Synthesis of a tetraketone of the tetracyclo[5.5.1.$0^{4,13}$.$0^{10,13}$]tridecane ("Staurane") series. J. Amer. Chem. Soc. 100, 3973-3974 (1978).

Chiang, C. C., DeCamp, W. H., Curtin, D. Y., Paul, I. C., Shifrin, S. and Weiss, U.: Color dimorphism of 14-hydroxy-morphinone. X-ray analysis of two different chemical modifications. J. Amer. Chem. Soc. 100, 6195-6202 (1978).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29003-12-LPC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The biological chemistry of membranes and complex membrane systems of biological interest.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Gerald M. Shean Research Chemist A:LCP

COOPERATING UNITS (if any)

Karl Sollner (Scientist Emeritus) A:LCP

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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1*	1*	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several findings were made concerning membrane hydrolysis across permselective porous membranes. The attainment of Donnan equilibria was more readily attained with protamine membranes than with sulfonated polystyrene membranes. One possible cause for this may be the slightly acidic solutions that routinely develop when aqueous solutions are exposed to air. The anticipated elevation of concentration potentials at low electrolyte concentrations was found to be true when the concentration ratio of one of the ions of water was made the same as that of the electrolyte, i.e., the Donnan distribution was present. A different type of membrane hydrolysis was shown to occur with a paraffin oil membrane. The mechanism depends upon the selective transport of undissociated electrolytes across the membrane.

*The principal investigator spent 5 months during the end of the last fiscal year and the beginning of this one on a detailed assignment to the Health Services Administration. An additional four months, 3/11/79 to 7/8/79, also were spent on a detail to the NIAMDD Extramural Program.

Objectives:

To study the physical chemical characteristics of liquid ion-exchange membranes, porous ion-exchange membranes, other membrane systems and models with the ultimate aim of providing a rational basis for various complex membrane phenomena in living systems.

Methods Employed:

The methods employed were essentially the same as reported previously, including flame photometry, potentiometric titrations (including the chloridimeter), spectrometry, non-aqueous titrations, radioactive tracer methods, etc.

Major Findings:

Experiments were performed to study membrane hydrolysis across permselective membranes when the ions of water distribute themselves on either side of the membrane in response to the Donnan membrane distribution that is required by electrolyte concentrations. As an example, with the cell (side 1) .1 N KCl | cation permeable membrane |.01 N KCl (side 2), the Donnan distribution would require that the activity (or roughly the concentration) of the hydrogen ions, from the dissociation of water, should be ten times more on side 1 than on side 2. Since the ionization product of H+ and OH^- is constant, the ratio of OH^- on either side of the membrane is the reciprocal of the H^+ ratio. Originally the H^+ activity would be about the same on side 1 and side 2, but the Donnan considerations cause a redistribution due to the permselective membrane. This redistribution of H^+ from side 2 to side 1 occurs by its exchange with K^+, which goes from side 1 to side 2. These factors influence concentration potentials across permselective membranes. The more dilute the electrolyte (KCl in the above cell), the more the ions of water interfere.

The experimental findings with cation permselective porous membranes made from sulfonated polystyrene and collodion, and with anion permselective porous membranes made from protamine and collodion demonstrated the Donnan effect, i.e., the ratio of H^+ or OH^- across the membranes after equilibrium was in many instances the same as the electrolyte ratio. The results were better for anion permselective membranes than for cation permselective membranes.

If the electrolyte concentrations are low, i.e., less than .005 N, the ions of water interfere with attaining theoretical maximum membrane concentration potentials. By adjusting the ratio of the acidity across cation permselective membranes, and the ratio of alkalivity across anion permselective membranes to match the electrolyte ratio, higher membrane potentials were attained.

A type of membrane hydrolysis not due to the same mechanism as that described above for porous membranes was demonstrated with a paraffin oil

membrane. When this separated sodium salicylate from water, the water side became more acidic and the sodium salicylate side became more alkaline. This was due to the transport through the membrane of undissociated salicylic acid.

Significance to Biomedical Research and the Program of the Institute:

Movement of ions through membranes and electrical potentials across them are important aspects of all life processes. The project tries to provide solidly founded, nonspeculative physico-chemical background information on the functional behavior of membranes in general. At the same time, these membrane studies have furnished a new, now widely used tool for the electrometric determination of ion activities--the use of permselective membranes as membrane electrodes. The work of recent years, such as model studies on the accumulation of electrolytes against concentration gradients, on the physical chemistry of liquid ion exchange membranes of extreme ionic selectivity, on carrier transport, and the successful prediction of certain involved effects in multi-ionic membrane systems, indicates that an increasing variety of complex membrane effects, similar to those in living nature, are becoming more and more amenable to a fairly complete physico-chemical analysis.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29004-10-LCP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Photochemical reactions and their mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Herman Ziffer	Research Chemist	A LCP
OTHER:	Ken-ichi Kawai	Visiting Associate	A LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to examine the effect of structure on the course of the oxa-diπ-methane rearrangement we are attempting to prepare 17β-hydroxy-5-(10)estrene-2-one by a new synthetic route. In an earlier study we showed that this compound did not yield the oxa-diπ-methane rearrangement product (a cyclopropyl conjugated ketone), however, as the result of latter studies we now wish to examine the sensitized (acetone) photochemistry of this compound.

This project is presently inactive.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29005-05-LCP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Asymmetric synthesis; structure, stereochemistry and NMR

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Herman Ziffer	Research Chemist	A LCP
OTHER:	Mitsuru Imuta	Visiting Associate	A LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Molecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A mixture of phosphate buffer, copper sulfate and pyridine was shown to catalyze an unusual cis-ring opening of several aryl oxiranes. The reaction was also used to introduce chloride, methoxide and hydroxide at the benzylic center of indene oxide, while adding a proton at the oxirane oxygen. A chiral modification of this reagent (copper sulfate-nicotine) was used for the asymmetric synthesis of (+)-1,2-indene oxide and (+)-cis-1,2-indanediol.

A series of substituted threo-R,R-diphenylethane diols, prepared by microbial reduction of the appropriate benzil derivatives, was converted to the corresponding substituted trans-(R,R)-stilbene oxides. The positive CD bands at 230-235 nm exhibited by these compounds agrees in sign and magnitude with those predicted from a model developed by Mason et al. for trans-stilbene oxide. Microbial reduction of p-methyl desyl chloride was shown to yield the threo-(R,R)-chlorohydrin, thus establishing a route for the preparation of optically active substituted cis-stilbene oxides.

The stereochemistry at the ring-junction of an aldol product from the reaction of 1-morpholino-1-cycloheptene and methyl vinyl ketone was determined by X-ray crystallography as trans.

Project Description

For a variety of studies in pharmacology and molecular biology it is very desirable and frequently essential to have both enantiomers of a compound under investigation in order to distinguish between specific and nonspecific effects. We have therefore extended our original studies on asymmetric synthesis to develop procedures: (1) for preparing compounds of high optical purity and known absolute stereochemistry and (2) for stereospecifically converting alcohols (products from our microbial studies) to amines and other derivatives. In asymmetric chemical reductions optical yields varied and the absolute stereochemistry of the products were unpredictable. However, in earlier studies we found that microbially mediated reductions of hydroaromatic ketones yielded alcohols of a predictable absolute stereochemistry and in high optical purity.

In some cases, e.g. if the ketone is also conjugated with a double or triple bond, little reduction occurs. Therefore, in addition to these reductions we examined the enantioselective hydrolyses of a series of acetates of racemic alcohols using a strain of Rhizopus nigricans. The alcohols formed in these hydrolyses were optically active with enantiomeric excesses in the 50-70 percent range. A consistent pattern of absolute stereochemistries about the carbinol carbons of unsubstituted cyclic and cyclic aryl alkyl carbinols was observed. A rule predicting the absolute stereochemistry of the enantiomer most readily hydrolyzed, based on the relative sizes of substituents on the carbinol, carbon was suggested which accounts for the observed results.

Proposed Course of Project

We plan to examine the enantioselective hydrolyses of several series of cyclic and acyclic esters in an attempt to test and define more explicitly the relative sizes of the substituents on the carbinol bearing carbon. In addition, some of the esters to be examined will be designed to test the scope and limitations of these enantioselective hydrolyses. If, as we hope, many classes of esters can be resolved by an enzyme(s) present in this organism we will attempt to isolate the enzyme and to attach it to a solid support.

We plan to continue to examine the scope and limitations of the copper-pyridine catalyzed oxirane ring opening reaction.

We also plan to prepare and examine several reactions of chromium-arene complexes. These will be prepared from optically active alcohols, obtained from microbial reactions, and used in a study of some asymmetric alkylations.

Publications

Imuta, M. and Ziffer, H.: Syntheses and absolute stereochemistry of cis- and trans-1,2-indane diols: metabolites of indene and 2-indanone. J. Org. Chem. 43: 4540-4542, 1978.

Imuta, M. and Ziffer, H.: Convenient method for the preparation of reactive oxiranes by direct epoxidation. J. Org. Chem. 44: 1351-1352, 1979.

Imuta, M. and Ziffer, H.: Syntheses and physical properties of a series of optically active trans-stilbene oxides. J. Org. Chem. In press.

Imuta, M. and Ziffer, H.: Novel copper-pyridine catalyzed ring opening reactions of aryl oxiranes: an asymmetric synthesis of (+)-indene-1,2-oxide and (+)-cis-1,2-indanediol. J. Amer. Chem. Soc. In press.

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REPORTED IN PRESS - 1978

Kabuto, K., Imuta, M., Kempner, E. S. and Ziffer, H.: Product stereospecificity in the microbial reductions of hydroaromatic ketones. J. Org. Chem. 43: 2357-2361, 1978.

Imuta, M. and Ziffer, H.: Microbial reduction of a series of substituted benzils, optical properties and NMR spectra of products. J. Org. Chem. 43: 3319-3323, 1978.

Imuta, M. and Ziffer, H.: Preparation and absolute stereochemistry of isomeric pyridylethanols and threo di-(2-pyridyl)ethanediol. J. Org. Chem. 43: 3530-3532, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29006-09-LCP

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The relationship between the nuclear and electronic configuration of molecules and their spectroscopic and dynamic properties.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Elliot Charney	Research Chemist	A:LCP
OTHER:	Che-Hung Lee	Staff Fellow	A:LCP

COOPERATING UNITS (if any)

Joan Rosenfield, NASA, Beltsville, Md. Kiwamu Yamaoka, Univ. of Hiroshima, Japan
I. Tinoco, Jr., Univ. of California, Berkeley
G.E. Manning, Dept. of Chemistry, Rutgers University

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Spectroscopy and Structure

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

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2.5	2	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A. Macromolecular structure, dynamics and polyelectrolyte properties of large biological polymers, in particular, polynucleotides are being studied by electric-field induced dichroism and birefringence methods.

B. Molecular chiral phenomena are being examined by quantum mechanicam calculations, (CNDO/S and CNDO/2), in organic compounds containing intrinsically dissymmetric conjugated systems.

PHS-6040
(Rev. 10-76)

Objectives:

I. Macromolecular Structure and Dynamics

The orientation of large polymeric molecules in pulsed electric fields and their disorientation when the field is turned off can be studied using monochromatic polarized light. These experiments are designed to obtain either the molecular conformation or structure in solution (from the dichroism, which is the difference between the changes in extinction of the light polarized parallel and perpendicular to the electric field when the molecules are completely oriented in the field) or the dynamics of intramolecular reorganization and of rotational disorientation (from the decay of the dichroism at the end of the field pulse). Both of these properties are significantly affected by the presence or absence of free charges on the polymer.

Having demonstrated over the past two years that the ionic environment of the biological polyelectrolytes, DNA and the synthetic polynucleotides poly(A) and poly(C), has profound effects on their orientation in electric fields and that at least two factors in this enviornment, the ionic strength of the counterions and the pH produce effects explicable in terms of rapidly developing polyelectrolyte theory, we have turned our attention to other environmental factors and to additional quantitive examination of these effects. The object is to use this information both to obtain structural information and additional clues to the mechanism of the electric field orientation. We have found, for example, that while increases in the ionic strength decrease the degree of orientation of these polyelectrolytes in the absence of buffer or in buffers containing monoionic species, when the buffer is a zwitterion which can bind to the charged polyelectrolyte, the orientation is almost independent of ionic strength and, in fact, has the inverse behavior (from unbuffered or monoionic species buffered) predictable from a recent theoretical analysis by Manning [Quart. Rev. Biophys. 11: 129 (1978)] of the polarizability of polyelectrolytes. We have also found that the effect of having a divalent charge counterion such as Mg^{++} present in solution rather than the monovalent Na^{+} is exactly as predicted by this theory. We are encouraged by these results to continue this work in order to provide critical tests of the available polyelectrolyte theories and to develop a new theory for the orientation mechanism based on these. We have, for example, underway a study in which the dielectric properties of the medium are varied in a known way. Since the counterion-counterion repulsion of the counterions bound to the polyelectrolyte is strongly affected by the dielectric constant of the intervening solution, it is expected that very profound effects will be observed.

The binding of intercalating dyes to DNA reported underway last year has been completed to the point of the analysis of the stoichiometry which we found deficient in studies reported by other investigators, but we have deferred utilizing these results to obtain structural information from electric dichroism studies because of the significance of the work reported above.

The conformations of the nucleic acids are subjects of intense study because of the role rather specific conformations are suspected to play in various replication processes. As an aid in investigating these, it has been helpful to try to determine and understand the conformation of di- and trinucleosides. NMR and a variety of other techniques have been used for this purpose, but frequently there are ambiguities in the interpretation of the data. We have decided to utilize a theoretical computation of the energies of some dinucleosides to determine which of several possible conformations of ApA and ApU are most likely to occur and to examine these for their accord with NMR data. Three stable structures very similar to those proposed by Lee and Tinoco [Biochemistry 16, 5403 (1977)] from visual examination of molecular models and NMR data, but with distinct structural variants, have been found. These structures provide still better accord with NMR data. An interesting feature of these calculations is that the structures are almost independent of electrostatic interactions between the bases but strongly dependence on the Van der Waal's interactions. The sequence-dependence of the three stable conformers has also been calculated.

Work has been started on the assembly of a new dichroism apparatus which will provide a higher radiation flux, deeper penetration into the ultra-violet and a faster response than our present apparatus.

II. Molecular Chiral Phenomena

A monograph which has been in preparation for several years has been completed.

In 1977 we [Rosenfield and Charney, J. Amer. Chem. Soc. 99, 3209 (1977)] published a report of the successful investigation of the quantum mechanical calculation of the effect of allylic substituents on the ultraviolet optical activity of dienes. In that publication it was pointed out that a particularly exacting test of the calculation would be the calculation of the circular dichroism of a substituted cyclohexadiene, α-phellandrene. The initial results, obtained by arbitrary assignment of structural parameters, was in reasonable qualitative accord with observations, but failed quantitatively. We have now applied the energy minimization of the Westheimer method utilized in the study of the dinucleoside conformation (vide supra) to obtain structural parameters and the conformational distribution dependence on temperature. Using these parameters, the recalculated values of the circular dichroism are in excellent quantitative agreement with the observations demonstrating the applicability of the energy minimization procedures for obtaining structural parameters in molecules of this type and the utility of the quantum mechanical calculation.

Proposed Course of the Project:

We will continue with the study of the structural and polyelectrolyte properties of macromolecules. In particular, we are now preparing a study of the dependence of the orientation on the charge density of the linear

polyelectrolyte. The first project in this study consists of an examination of the relative orientations of simple stranded poly(A), double-stranded poly(A)·poly(U) and triple-stranded poly(A)·poly(u)·poly(U) in which the charge density varies in the ratio 1:2:3. The work now underway on the structure of DNA in water-alcohol mixtures and on the relation of the field-induced orientation in these mixtures to polyelectrolyte properties reported above will be continued.

Publications:

Charney, E.: Dependence of the electric field-induced orientation of poly (riboadenylic acid) on its polyelectrolyte properties. Macromolecules 11: 1059-1060 (1978).

Charney, E.: Electric-field induced orientation of polynucleotides. In Jennings, B. R. (Ed.): Proceedings of the Conference on Electro-Optics and Dielectrics of Macromolecules and Colloids. Plenum Publishing Corp., 1979, pp. 149-152.

Charney, E.: The molecular basis of optical activity, John Wiley-Interscience (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29007-08-LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structure and interaction of paramagnetic biomolecules.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Hideo Kon Research Chemist A:LCP

OTHER: Fumihide Inoue Visiting Fellow A:LCP

COOPERATING UNITS (if any)

R.H. Menard (H-LCPO) K. M. Smith (Univ. of California, Davis)
N.R. Bachur (C-BCRC) S. Matsuura (CH-ERRB)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Spectroscopy and Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

EPR studies of dimerization of Cu(II)porphyrins have been extended to water soluble porphyrin Cu(II)tetra(4-sulfonatophenyl)porphyrin, and further information was obtained for the chemical conditions of dimerization in Cu(II)-cytochrome and its heme peptides. Also, dimer formation of high spin Fe(III)-porphyrins, other than μ-oxo bridged type was established for the first time; the structural characteristics of the dimers and the chemical environment of formation were clarified.

PHS-6040
(Rev. 10-76)

Project Description:

The purpose of this particular phase of the project is to investigate the structural factors and chemical conditions for the dimer formation of metalloporphyrins, specifically, in Cu(II) and Fe(III) high spin porphyrins and porphyrin containing polypeptides. Magnetic measurements were made mainly by using EPR (electron paramagnetic resonance) spectroscopy and Faraday balance susceptometer as an auxiliary means, both at cryogenic temperatures. The EPR signals which are suspected of belonging to a dimer species were studied by varying the chemical environments to obtain evidence, and the assignment further confirmed by quantum mechanical calculations of expected signal characteristic as well as by computer simxlations of the line shape.

Major Findings:

1. Cu(II)porphyrins: Continuing from 1978 report, dimer formation of water soluble Cu(II)tetra(4-sulfonatophenyl)porphyrin(I) was investigated. Such meso-aryl substituted porphyrins with net negative charge have been generally considered not to form dimer due to the steric hinderance as well as the electrostatic repulsion. Recent NMR and T-jump studies, however, indicated some distant dimer formation, but no structural information was available. We have found that (I) forms a definite tight dimer at pH 7, and we have determined the Cu-Cu distance (4.9Å) and the angle between Cu-Cu axis and the normal to the plane to be 33°. In contrast, similar but non-water soluble meso-aryl porphyrins was found not to form such a tight dimer. The distinction comes from the fact that in the latter the solvation takes place mainly on the porphyrin plane preventing the π-π type interaction, whereas in the water soluble analogue hydration takes place exclusively around polar end groups.

In further studies of Cu(II) substituted cytochrome c and its octa- and undeca peptide, it was found that (i) there is less dimer formation in the molecule with a longer side chain when the chain is a random coil, whereas in cytochrome c, the protein moiety appears to retain some definite conformation favoring dimer formation, and (ii) imidazole coordination and dimer formation are not mutually compatible.

2. Fe(III) high spin porphyrins: Aggregation of heme (hemin) in aqueous solution is an old problem, but very little has been clarified for the conditions of specific dimer formation and its structure, especially in Fe(III) high spin porphyrins. By studying a model compound (e.g., Fe(III)Cl-(meso-nitro-octaethylporphyrin) in organic solvent, we have found well-defined concentration dependent EPR signals in addition to the well-known absorptions from high spin Fe(III) porphyrin monomer. Quantum mechanical calculations based upon a dimer model confirmed the assignment of one of the two signals to a dimeric species. The other signal behaves as if it is due to a higher order polymer. The nature of the interaction in the dimer is consistent with π-π type, and the spin-spin exchange interaction is less than ~ 0.1 cm^{-1}, much smaller than in similar Cu(II) dimers. Extending the study to hemin in aqueous solutions, an analogous signal was found always whenever the medium

contains some hydrophobic component. In addition, hemin and water soluble Fe(III) Na_4(tetra-p-sulfonatophenylporphyrin) in an alkaline solution shows another extra signal located close to the g=6 monomer absorption. This is interpreted as due to a distant dimer much longer in Fe - Fe distance ($\sim$ 7Å) and a large skew angle (60°).

Thus we have found evidence that the μ-oxo bridged dimer, which has always been assumed whenever dimerization of Fe(III) porphyrins is demonstrated, is not the only type, but there are other dimeric species which should be taken into account to explain the physical properties of Fe(III) porphyrin solutions, and we have defined the chemical conditions for their formation.

Proposed Course of Project:

EPR studies of a series of metalloporphyrins in solution will end shortly with addiional one or two more systems, i.e., NO liganded Fe(II)porphyrins. EPR studies of biomolecules will continue with the emphasis of use of spin labels. In particular, preparation is underway to study the effect of the skeletal protein cross-linking on the fluidity of red cell membranes.

Publications:

Chikira, M. and Kon, H.: EPR evidence for dimer formation of Cu(II)tetra(4-sulfonatophenyl)porphyrin sodium salt in aqueous solutions. Heterocycles, 11: 253-259 (1978).

Menard, R.H., Guenthner, T.M., Taburet, A.M., Kon, H., Pohl, L., Gillette, J.R., Gelboin, H.V., and Trager, W.F.: Specificity of the in vitro destruction of adrenal and hepatic microsomal steroid hydroxylases in thiosteroid. Mol. Pharma (in press).

REPORTED IN PRESS - 1978

Chikira, M., Kon, H., Hawley, R.A., and Smith, K.M.: ESR study of dimers of Cu(II)-Octaethylporphyrin and the meso-nitro and meso-di-nitro derivatives in solution. J.C.S. Dalton, 245-249 (1979).

Chikira, M., Kon, H., and Smith, K.M.: ESR evidence for dimer formation in high spin Fe(III) octaethylporphyrin and meso-nitro-octaethylporphyrin. J.C.S. Chem. Comm., 906-907 (1978).

Rispin, A.S., Sato, M., and Kon, H.: Crystal field calculation of g values and zero-field splitting for high spin ferric complexes of rhombic character. Theor. Chim. Acta, 50: 95-116 (1978).

Bachur, N.R., Gordon, S.L., Gee, M.V., and Kon, H.: NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. Proc. Nat. Acad. Sci. USA, 76: 954-957 (1979).

Menard, R.H., Guenthner, T.M., Kon, H., and Gillette, J.R.: Studies on the destruction of adrenal and testicular cytochrome P-450 by spironolactone. J. Biol. Chem., 254: 1726-1734 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29008-08-LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Electronic and molecular structural investigations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Ruth McDiarmid Research Chemist A:LCP

COOPERATING UNITS (if any)

John Doering - Johns Hopkins University

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Spectroscopy and Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. The ultraviolet spectra of ethylene, deuteroethylene and all five partially deuterated ethylenes were measured between 1210 and 1600Å and analyzed. The resultant assignments differ somewhat from prior assignments. As a result of this investigation, ethylene is now one of the few polyatomic molecules in which 4 Rydberg series, including 2 d-series, are observed.

2. The remainder of the year's effort was spent setting up a laboratory for multiphoton ionization spectroscopy of cooled molecular beams.

PHS-6040
(Rev. 10-76)

Project Description:

The aims of this research are to engender a better understanding of the electronic structure of molecules and to provide an experimental assessment of the progress of theoretical models of and calculations on molecules. To understand the structure of molecules, we seek to know, among other factors, the energies of and energy differences between different electron orbitals, the geometric rearrangements accompanying electron promotion, and the inductive effects and/or indirect bonding effects of one chemical group on another to which it is bonded. To attain these goals, we are studying electronic absorption spectroscopy and multiphoton ionization spectroscopy. Electronic spectroscopy directly provides information about the energy differences between orbitals and indirectly provides geometric information. Multiphoton ionization spectroscopy both enables optically forbidden transition to be observed and permits the observation of very small quantities of material.

In the investigation summarized above, transitions to s, p, $d\sigma$ and $d\delta$ Rydberg orbitals were observed and the previously observed disparity between the spectra of the ethylene ion and the lowest energy Rydberg transition was resolved. These results, in conjunction with those obtained in the past on substituted ethylenes, now enable a systematic analysis to be made of the effect of substitution on the electronic spectrum of ethylene, the simplest unsaturated molecule. This investigation will be pursued.

Past work on the electronic spectrum of butadiene is being extended in a recently initiated investigation of its electron impact spectrum in collaboration with Dr. John Doering of Johns Hopkins University. Electron impact spectroscopy has different selection rules than optical spectroscopy. This investigation is intended to resolve some of the residual ambiguities and controversies in the analysis of the spectrum of butadiene. Preliminary measurements and analysis suggest that the broad optical $\pi \rightarrow \pi^*$ peak contains two electronic transitions, one of which is optically forbidden. The electron spectrum of the higher energy region to the blue of the $\pi \rightarrow \pi^*$ system indicates that some of the optical transitions are allowed and some are forbidden. The detailed analysis of this data is in progress.

In the next year, in addition to the above, we plan to initiate a series of investigations of the multiphoton ionization spectra of very cold gaseous molecules. Instrumentation development and testing has already started and the system should soon be operating. Initial efforts will be focussed on previously investigated molecules--ethylene, butadiene, dimethylsulfide--both to calibrate the new system, to locate transitions not observable in the optical spectra, and to resolve problems remaining in the interpretation of the optical spectra. Once types of optical results obtainable on the new experimental apparatus have been characterized, we will investigate the limitations on the types of molecular systems that can be studied with the new experimental apparatus. These studies are anticipated to continue for several years.

Publications:

REPORTED IN PRESS - 1978

McDiarmid, R.: Transition in the electronic absorption spectra of *cis*- and *trans*-difluoroethylene, J. Chem. Phys., 69: 2043-2053, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29009-06-LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies on sickle cell disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William A. Eaton	Medical Director	A:LCP
OTHER:	James Hofrichter	Research Chemist	A:LCP
	Frank A. Ferrone	Staff Fellow	A:LCP
	Helen R. Sunshine	Staff Fellow	A:LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Macromolecular Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The thermodynamics and kinetics of hemoglobin S gelation are being investigated in order to develop an inhibitor that could be used as a therapeutic agent for sickle cell disease.

HHS-6040
(Rev. 10-76)

Objectives:

The major aim of this work is to elucidate the molecular mechanism of hemoglobin S gelation in order to provide a rationale for the development of agents that can be used in the treatment of patients with sickle cell disease.

Methods Employed:

In addition to conventional biochemical methods, a variety of specially designed physical techniques have been employed to study the gelation process. These include microspectrophotometry, turbidity, linear dichroism and birefringence, water protein NMR linewidths, temperature perturbation kinetics, laser photolysis, and laser light scattering.

Major Findings:

I. Molecular Mechanism of Hemoglobin S Gelation

This study is aimed at providing a theoretical model for the molecular mechanism of gelation that makes quantitative predictions of the kinetic behavior, and yields parameters that can be interpreted in terms of molecular structure.

We have previously described a simple equilibrium nucleation theory which can rationalize the extreme sensitivity of the delay time to solution variables, and which accounts for the close interrelation between the kinetics and the thermodynamics of gelation. In this model the critical nucleus is the thermodynamically least stable species and is treated as a constant. We have begun the development of a more realistic theory, which allows for the change in the size of the critical nucleus with increasing concentration. The major uncertainties in the theory are (i) the applicability of the ideal gas equations for calculation of the loss of rotational and translational entropy upon aggregation, (ii) the magnitude of the vibrational entropy of the aggregate that results from intermolecular modes, and (iii) the accuracy of the estimates for the activity coefficient of the activated complex of the critical nucleus. As a result, it is not yet possible to predict whether the concentration dependence of the delay time will increase with increasing concentration from the effect of nonideality, or decrease with increasing concentration from the decrease in nucleus size. The photolysis technique, described in II of this report, has been developed in part to provide an experimental answer to this question.

II. Photoinduced Gelation of Carbomonoxyhemoglobin S

The kinetics of the thermally induced gelation of hemoglobin S have been studied extensively in the time range 10^2 to 10^5 seconds. In order to investigate the kinetics of much faster gelation, we have developed a technique which employs photodissocation of carbon monoxide from HbCO S to initiate polymerization. The formation of polymers is detected by light scattering from the same laser that is used as the photolysis source. To

understand the experimental method in quantitative detail, we have investigated the rate of diffusion of carbon monoxide out of the photolysed volume, the dependence of the fractional desaturation on laser power density and optical configuration, and the effect of heating from the large amount of light absorption. All of these effects are now well understood in terms of the independently determined parameters of the system, which include the photodissociation quantum yield, the rate of carbon monoxide recombination, the diffusion of carbon monoxide in concentrated hemoglobin solutions, and the heat conductivity of the sample.

The principal results were (i) the concentration dependence of the delay time decreases continuously from about 35th power at 25 g/dl to about 15 at 35 g/dl, (ii) the apparent activation energy behaves very similarly to that observed for the thermally induced process, (iii) the time dependence decreases from powers >10 for progress curves with delay times greater than 10 seconds to about 3 to 4 for progress curves with delay times of about 0.01 seconds, and (iv) the delay times at physiological concentrations can be much less than capillary transit times, lending support to the kinetic hypothesis formulated in this Laboratory for the pathophysiology of sickle cell disease. (See Hofrichter's annual report for a more detailed description of this project.)

III. Oxygen Binding and Hemoglobin S Gelation

One of the major unexplored problems in sickle hemoglobin gelation is the influence of oxygen on the thermodynamics and kinetics of the process. The principal objective of this project is to provide a thermodynamic description of the effect of oxygen by measuring the fractional saturation of both the solution and polymer phases as a function of total hemoglobin concentration and oxygen partial pressure.

The initial goal has been to solve some major technical problems associated with the measurements. First, the near infrared spectra have been characterized in detail in order to obtain an accurate set of extinction coefficients for the oxy, deoxy, and met forms. Second, pilot measurements on gels formed from partially saturated solutions showed that methemoglobin formation is much too rapid, and severely interferes with the measurements. In order to solve the oxidation problem an enzyme system is being developed which reduces methemoglobin to deoxyhemoglobin, and simultaneously minimizes oxygen consumption.

IV. Comparison of Potential Therapeutic Inhibitors

Since the discovery that intracellular gelation is responsible for sickling, a central idea in sickle cell disease research has been that partial inhibition of intracellular gelation should decrease clinical severity, while complete inhibition should effect a "cure" of the disease. More recently we have proposed that the inhibition should be kinetic, and have formulated a pathophysiological mechanism in which the delay time of intracellular gelation, relative to the capillary transit time, is the primary determinant of clinical

severity. This kinetic hypothesis immediately suggests that increasing the delay time will result in amelioration of the disease. We have also described a set of criteria for judging the therapeutic potential of an agent by correlating the clinical severity of sickle cell disease with an in vitro delay time measurement. The criteria are based on delay time measurements for hemoglobin S in mixtures with normal adult and fetal hemoglobins.

The major objective of the current study was to compare the effects of the most well-characterized inhibitors - urea, butylurea, phenylalanine, and cyanate - on the in vitro delay time of gelation. Several important results emerged from this comparison. First, the effect of urea is predicted to be at least 5 times too small to have a detectable therapeutic effect in patients. Phenylalanine is a much more powerful inhibitor, but the concentrations required to give some therapeutic effect are 10 to 20 times higher than the levels which are known to be toxic in children from studies on phenylketonuria. Cyanate is by far the most effective inhibitor on a mole ratio basis. Including the effect of cyanate at in vivo carbamylation levels is predicted to decrease the clinical severity to approximately the clinical status of a patient with sickle/β^+-thalassemia, which is exactly what was observed in the clinical trials. This result provides strong support for the quantitative predictions of the kinetic hypothesis.

Significance to Biomedical Research and the Program of the Institute:

Our kinetic studies constitute a totally new approach to understanding the pathophysiology of sickle cell disease and to the development of therapeutic measures.

The theoretical approaches, the experimental results, and the methods developed in this investigation should contribute to a general understanding of other macromolecular self-assembly processes.

Proposed Course of Project:

A systematic investigation of the photoinduced polymerization of HbCO S will be continued to describe the kinetic behavior over a wide range of delay times, concentration, and temperature. Using this technique the distribution of intracellular gelation delay times will be determined for whole blood samples, and the effect of potential drugs on this distribution will be investigated. Theoretical models for the molecular mechanism of gelation will be explored both analytically and numerically. The effect of oxygen on gelation will be investigated by determining the composition of the solution and polymer phases of a gel over a wide range of conditions.

Publications:

Ferrone, F.A., Hofrichter, J. and Eaton, W.A.: Polymerization of sickle hemoglobin in the photostationary state. In *Frontiers of Biological Energetics, Volume II: Electron to Tissues*, P.L. Dutton et al. (Eds.), (Academic Press, New York, 1978), pp. 1085-1092.

Sunshine, H.R., Ferrone, F.A., Hofrichter, J., and Eaton, W.A.: Gelation assays and the evaluation of therapeutic inhibitors. In *Development of Therapeutic Agents for Sickle Cell Disease*, J. Rosa (Ed.), (Elsevier/North Holland Biomedical Press, Amsterdam, 1979), pp. 31-46.

Sunshine, H.R., Hofrichter, J., and Eaton, W.A.: Gelation of sickle hemoglobin in mixtures with normal adult and fetal hemoglobins. J. Mol. Biol. (in press).

REPORTED IN PRESS - 1978

Eaton, W.A. and Hofrichter, J.: Successes and failures of a simple nucleation theory for sickle cell hemoglobin gelation. In *Biochemical and Clinical Aspects of Hemoglobin Abnormalities*, W. S. Caughey (Ed.), (Academic Press, New York, 1978), pp. 443-457.

Sunshine, H.R., Hofrichter, J. and Eaton, W.A.: Requirements for therapeutic inhibition of sickle haemoglobin gelation. Nature, 275: 238-240 (1978).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29010-07-LCP

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Conformation and electronic structure of biological molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William A. Eaton	Medical Director	A:LCP
OTHER: James Hofrichter	Research Chemist	A:LCP

COOPERATING UNITS (if any)

Robin M. Hochstrasser, Univ. of Pennsylvania

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Macromolecular Biophysics

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The electronic structure of retinal is being investigated by polarized single crystal absorption spectroscopy. The dynamics of structural transitions in hemoglobin following photodissociation of the carbon monoxide complex are being investigated with a transient laser spectrometer.

PHS-6040
(Rev. 10-76)

Objectives:

The aim of this research is to (i) relate the optical properties of biological molecules, particularly proteins with chromophoric prosthetic groups, to their electronic and geometrical structure, and (ii) use this information to provide an experimental description of the dynamics of conformational transitions in proteins using time-resolved pulsed laser spectroscopy.

Methods Employed:

In addition to conventional biochemical and spectroscopic methods, a general phase modulation microspectrophotometer has been designed and constructed by Dr. Hofrichter for measuring a variety of steady state anisotropic optical properties of microscopic samples, including polarized absorption, linear and circular dichroism, and linear and circular birefringence.

For interpretation of spectral results in terms of assignments we have used the self-consistent charge, extended Hückel LCAO-molecular orbital method developed by Gouterman and coworkers at the University of Washington.

For measurement of transient absorption, linear dichroism and birefringence, luminescence, and Raman scattering, an excite and probe type spectrometer is being developed, which is based on two Nd:YAG nanosecond lasers, a dye laser, and an optical multichannel analyzer.

Major Findings:

I. Optical Spectra of All-trans Retinal (in collaboration with Dr. Hofrichter and Dr. Hochstrasser)

Although the electronic spectroscopy of retinals has been studied for many years, the series of weak transitions predicted by theory have not been observed and there is no quantitative data at all for the directions of electric dipole transition moments. We have begun an investigation of the room temperature polarized single crystal absorption spectrum of all-trans retinal, the final photoproduct of the visual excitation process.

Ultra thin crystals (<200Å) were grown from solution between closely spaced quartz flats. The crystal spectra were measured on the 101 face. Because the crystal became progressively photodamaged with irradiation by the measuring beam, the spectra were measured as a function of time to insure that the initial spectra were not significantly influenced by the photoproduct spectrum. In contrast to the solution spectrum, which contains only a single broad absorption band between 500 nm and 230 nm, the crystal spectrum showed four overlapping bands in this region. We label these four bands I - IV, in order of decreasing wavelength. Bands I and IV were about 1/10th as intense as bands III and IV, which had roughly equal intensity. Together it would appear that bands III and IV underly the single intense band of the solution

spectrum. The polarization ratios for all four transitions were very similar, indicating similar directions (to within a few degrees) for the electric dipole transition moments. The exciton splitting for band II was clearly resolved and was $\sim$1500 cm^{-1}, while that of band III was less than half the magnitude and of opposite sign. This result indicates that although bands II and III have about the same intensities and transition moment directions, they have substantially different excited state electron distributions.

We have not yet fully analyzed the results in terms of detailed theoretical predictions, so that our assignments are only tentative. Band I is most probably the forbidden "A_g" state described by Kohler and coworkers, which is very important for understanding the photochemistry of retinals. Band IV may be the "cis" band that is effectively forbidden in all-trans retinal. Bands II and III strongly overlap in the crystal spectrum, but are resolved because of their differing exciton splittings. One possibility is that band II corresponds to the main band of the solution spectrum, which is the allowed $\pi \rightarrow \pi^*$ transition. Band III, then, is presumed to be weak in the free molecule and not observed in the solution spectrum. In the crystal, however, intermolecular interactions result in intensity transfer from band II to III. This possibility is currently being explored from theoretical calculations.

II. Molecular Mechanism of Structural Changes in Hemoglobin Upon Release of Oxygen and Carbon Monoxide (in collaboration with Dr. Hofrichter)

Upon dissociation of oxygen or carbon monoxide, hemoglobin undergoes a complex series of tertiary and quaternary structural changes that are responsible for its cooperative behavior. Among these are the doming of the porphyrin with a displacement of the iron from the heme plane, a reorientation of the heme associated with conformational changes in the surrounding globin, a quaternary structural change that results from alterations in the intersubunit bonding, and finally a further intrasubunit conformational change imposed by the constraints of the new (T) quaternary structure. The general objective of this project is to use newly developed pulsed laser techniques to describe the dynamics of these processes, beginning with the dissociation of ligand.

During the past year we have made detailed plans for the assembly of an excite and probe type spectrometer, based on 2 nanosecond Nd:YAG lasers. The pulse from one laser will be used to photodissociate HbCO or HbO_2, which is known from our previous study to take no more than a few picoseconds at room temperature. The electronically delayed probe pulse will then be used to interrogate the solution. By using a spectrograph and optical multichannel analyzer, it will be possible to measure a complete absorption spectrum within the time width ($\lesssim$10 ns) of a single laser shot. A more detailed description of the transient laser spectrometer is given in the annual report by Hofrichter.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the electronic structure and mechanisms of conformational transitions in proteins should lead to a much deeper understanding of how these molecules perform their biological function. The techniques developed in our investigations may be applied to a wide variety of problems in protein structure and chemistry.

Proposed Course of Project:

In the coming year we will assemble the components of the transient laser spectrometer and begin our investigation of the dynamics of the structural changes in hemoglobin.

Publications:

Greene, B.I., Hochstrasser, R.M., Weisman, R.B., and Eaton, W.A.: Picosecond transient absorption spectra of hemoglobin. In *Frontiers of Biological Energetics, Volume II: Electrons to Tissues*. P.L. Dutton et al. (Eds.), (Academic Press, New York, 1978), pp. 1075-1083.

REPORTED IN PRESS - 1978

Eaton, W.A., Hanson, L.K., Stephens, P.J., Sutherland, J.C. and Dunn, J.B.R.: Optical spectra of oxy- and deoxyhemoglobin. J. Amer. Chem. Soc. 100: 4991-5003 (1978).

Greene, B.I., Hochstrasser, R.M., Weisman, R.B. and Eaton, W.A.: Spectroscopic studies of oxy- and carbonmonoxyhemoglobin following pulsed optical excitation. Proc. Nat. Acad. Sci. USA 75: 5255-5259 (1978).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AM-29011-08-LCP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Physics and Chemistry of Photoreception

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William A. Hagins	Medical Officer	A LCP
OTHER:	W. E. Robinson	Research Biologist	A LCP
	S. Yoshikami	Research Biologist	LVR EI
	F. M. Hagins	Guest Worker	A LCP
	H. Kon	Research Chemist	A LCP
	M. Rashidian	Electronics Engineer	A LCP
	J. Berman	Summer Employee 25 February-30 September 1979	A LCP
	J. George	Biological Laboratory Technician	A LCP

COOPERATING UNITS (if any)

W. Banfield, Medical Officer, LP C

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Membrane Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An investigation of the mechanism of visual excitation in vertebrates.

PHS-6040
(Rev. 10-76)

Project Description:

During 1978 several important steps were taken in the project to identify the intracellular transmitter of visual excitation in retinal rod cells. Methods were developed for loading known high concentrations of a calcium-sensitive indicator dye or of the metal buffer, EDTA, into outer segments of living retinal photoreceptor cells (Yoshikami, Hagins). Attempts to show transient light-induced changes in intracellular calcium concentrations gave negative results. At the same time, rods loaded with large amounts of EDTA did not show the diminished light responses that would be expected if free Ca^{++} was the excitatory transmitter substance. However, it could be shown that Ca^{++} displaced from the intracellular EDTA by Co^{++} ions was as effective as light in suppressing the sensory "dark current" of rods. It is possible that the similarity of action of Ca^{++} and light in controlling the dark current arises from an indirect effect of Ca^{++} on the intracellular concentration another substance which is itself the actual sensory transmitter.

A new method has been developed for rapidly isolating intact rod outer segments and studying the effects of light, Ca^{++}, and a wide range of drugs on the metabolism of cytoplasmic nucleotides (Robinson, Hagins). The aim of the method is to study light induced changes in energy metabolism in rod outer segments. It has been shown that outer segment cytoplasm contains about equal amounts of GTP and ATP but the GTP:GDP ratio is about 1, while the ATP:ADP ratio is 4-8, a value much closer to that of healthy brain cells. Much of the GDP in outer segments is bound to sites that are nearly as abundant as rhodopsin molecules themselves. When freshly isolated outer segments are exposed to a flash that photoisomerizes 1% of their rhodopsin, three biochemical events take place

A) About 1 mmol of GDP per liter of outer segment volume becomes inextractable within less than 4 sec.

B) 80% of the 4-5mM GTP in outer segments is converted to GDP with a half-time of 10 sec. At the same time, an equivalent quantity of creatine phosphate disappears from the rod cytoplasm. There was no change in adenine nucleotides.

C) After 60 sec. a small amount of GMP is formed, perhaps equivalent to 15% of the GTP consumed.

D) There is a 20-30% fall in the 50μM concentration of extractable 3'-5' cyclic GMP in outer segments within 1 min of the bleaching flash.

We conclude from the above observations that light causes marked activation of GTP hydrolysis by structures in rod outer segments. It is not yet clear whether the products are GMP and pyrophosphate (as would be expected from a cyclose-phosphosphodiesterase enzyme system known to exist in outer segments) or a specific GTPase. It is clear, however that large amounts of biochemical free energy are mobilized in rod outer segments when light is absorbed in them. This free energy may well be a part of the cycle of release and removal of the excitatory transmitter. We intend to pursue the study of metabolic events during visual excitation.

Publications:

Robinson, W. E. and Hagins, W. A.: GTP hydrolysis and the transmitter cycle in visual excitation. Nature. In press.

Hagins, W. A.: Excitation in vertebrate photoreceptors. Fourth Neurosciences Study Program, Boulder, Colo. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29012-09-LCP

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

The influence of molecular structure on chemical and biological properties

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Norman E. Sharpless	Research Chemist	A LCP
OTHER:	Ralph G. Adams	Research Physicist	A LCP
	Frederick S. Brackett	Scientist Emeritus	A LCP
	William H. Jennings	Research Physicist	A LCP
	Peter F. Kador	Visiting Fellow	EI LVR
	Ulrich Weiss	Scientist Emeritus	A LCP

COOPERATING UNITS (if any)
C. H. L. Kennard — Reader in Chemistry, University of Queensland, Brisbane, Australia

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Membrane Biophysics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The energy minimization calculations on various compounds of biological interest have given insight into their behavior and clues to the possible pharmacophores. Cluster analysis on the retinal isomers shows three categories due to energy and conformational variations, which have been related to biological behavior.

The inhibition of aldose reductase by flavonoids and related compounds has been extended to include the chromon-2-carboxylic acids, as well as other related compounds.

PHS-6040
(Rev. 10-76)

Project Description:

This project investigates the molecular and submolecular factors, such as structural features, quantum parameters and physical properties in relation to biological and pharmacological behavior of biologically important substances.

Energy minimization and conformation studies.

Using the program GEMO, continued studies on the relative stabilities and conformations of the retinal isomers have been carried out to gain insight into the visual process. This has permitted the calculation of molar extinction coefficients for previously unavailable 7-cis isomers of retinene (7,13-dicis and 7,11-dicis).

Recent studies indicate that the visual process also involves the 11,12-s dicis rotamer of retinene, which undergoes photochemical transformation into the 12-s cis rotamer, followed by thermal isomerization into the bicis form. The energy differences show this to be feasible. Preliminary calculations indicate this rotational barrier to be in the 40-50 kcal/mol range.

Cluster analysis of the retinene isomers, based on both energy and shape factor considerations, shows, that for shape considerations, the 11,12-s, the 11-s and the trans isomers are unique; the same relationship tends to hold when energies are considered. Thus both energetics and pharmacophore parameter are involved in the visual process.

Aldose reductase has been implicated in the events which lead to sugar cataracts by its action in forming sugar alcohols, which may be causes of the cataracts. Flavonoids have been reported to be potent inhibitors of aldose reductase. The postulated mechanism of inhibition is a nucleophilic assault on the carbon of a carbonyl group by the reductase, which thus becomes unavailable to its normal substrate. Since certain stereochemical requirements must also be met, a new series of related compounds, the 5- or 7- substituted chromone-2-carboxylic acids have been investigated by quantum calculations and energy minimization studies. This action of the flavonoids is a function also of the energy of the lowest empty molecular orbital of the molecule. As a consequence of this finding a large number of possible compounds has been screened by molecular orbital calculations to spot those which may have favorable values of LEMO. The two compounds having the best possible values are nitrogen analogs of the chromones. Attempts are now underway to obtain these by synthesis. Other compounds investigated as aldose reductase inhibitors are the 7-fluorohydantonyl chromones. These compounds can theoretically exist in 8 conformations (Boat or Chair; D or L). The strain energies of this series range from 67 to 100 kcals/mol, although the LEMO's are nearly constant. Since only one form is active, the principal factor differentiating them is molecular shape. This is being further investigated utilizing strain energy studies on N-substituted naphthalene-1,8-dicarboxylimides.

Additional studies which indicate that stereo factors are important are the tricyclic antidepressants related to imipramine. Strain energies in these compounds vary over a wide range, but certain shape factors remain constant.

Additional studies are being carried out on very highly strained molecules, such as fenestrane and staurane.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29013-08-LCP

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

The physics and chemistry of the visual pigments

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ralph G. Adams	Research Physicist	A LCP
OTHER:	Norman E. Sharpless	Research Chemist	A LCP
	William H. Jennings	Research Physicist	A LCP

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Model systems of visual pigments are constructed using retinal and retinal isomers in π-π complexes with amino acids and peptides. Models are examined both spectroscopically and with computer programs (GEMO) applications to determine how to approximate rhodopsin.

PHS-6040
(Rev. 10-76)

Project Description

The aim of my current research is the characterization of the structure of rhodopsin, primarily mammalian. Although there are other constituents, consideration is given mainly to the chromophore and the proteinaceous portion of the molecule. The ultimate aim is the reconstruction of rhodopsin in vitro.

By late June of 1978 we had rather convincingly (I think) established, by test tube experiments and by computer data, that the tripeptide α-acetyl-l-lysyl-l-alanyl-l-phenylalanine methyl ester is a good guess as the attachment point of retinal in rhodopsin (the acetyl and the ester groups being replaced by amino acids). At that time new information about the amino acid sequence of opsin (the rhodopsin protein) showed that the tripeptide actually was reversed and should be l-phenylalanyl-l-alanyl-l-lysine. Calculations (ours) show this is non-trivial. All previous work had to be repeated and is nearly complete.

Synthesis of the reversed tripeptide has been accomplished but purification procedures are wasteful and until now only a small amount is available. The results obtained from limited experiments are little different from previous data using the reversed tripeptide but more work is obviously needed.

Characterization of the energy states of all-trans retinal (which is actually 6-s-cis) led to an answer to a question crystallographers have had regarding an uncertainty in the position of a ring carbon in retinal. After looking at the computer data (GEMO) it becomes apparent that there are two possible positions of the carbon in question with almost a 50-50 ratio. The result is that crystal measurements can never precisely show position except as an average.

Cluster theory, for which we had great hope, has thus far not produced much useful data but deserves some further effort.

For other progress see Sharpless.

Future Work

1. We need expansion of the GEMO program to allow larger molecules for determination of how rhodopsin should be assembled especially since the amino acid sequence is now known.

2. Test tube work has fallen far behind the computer data and needs doing for verification.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29014-19-LCP

PERIOD COVERED
October 1, 1978, through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Effects of environmental stress (hypoxia, exercise, smoke) on animals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paul D. Altland	Research Physiologist	A LCP
OTHER:	Milton G. Parker	Biological Lab Technician	A LCP

COOPERATING UNITS (if any)
Barnett Rattner (Guest Worker), Patuxent Wildlife Research Center

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Section on Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In a study of the potential prophylactic effects of long-term conditioning to various environments on the susceptibility of mice to decompression sickness it was found that treadmill exercise training enhanced decompression survival. Decompression depths of 7.6, 8.7, or 9.9 Atmospheres Absolute (pO_2=0.5 ATA) with N_2 were used. Exposure to cold (4°C-4hr/d-28 d) or hypoxia (P_B 379 Torr-4hr/d-28 d) or swim exercise (15 min/d in 31°C water-28 d) prior to decompression induced either no change in decompression survival rate (cold or swim exercise) or reduced survival rate (hypoxia). Enhanced survival in the Treadmill Exercise Group is presumably due to lean body confirmation and superior cardiovascular and respiratory fitness, whereas increased bends incidence in the Hypoxia Group may be attributable to greater blood viscosity due to polycythemia and hemo-concentration.

PHS-6040
(Rev. 10-76)

Project Description:

Many studies have been conducted to delineate procedural, environmental, and physiological factors that affect tolerance to decompression sickness. Some of the factors which affect susceptibility to decompression sickness include age and somatotype: however, the notion that bends susceptibility is associated with degree of physical fitness has not been established. Adaptation to hot environments or high altitude residence has been reputed to increase the resistance of men to a variety of unfavorable environmental factors such as acceleration, high temperature, hypoxia, and heavy physical exercise, but the principle of cross-adaptation is not well understood. We have studied the cross-adaptive effects induced by long term exposure to cold, hypoxia, or physical training on hyperbaric decompression tolerance in mice. Physical training induced by 28 daily 1 hour treadmill exercise sessions dramatically reduced the incidence of bends in decompressed mice. Apparently fitness, in addition to age and somatotype, is also associated with susceptibility to decompression sickness. The increased resistance brought about by training was not solely caused by a reduction of body weight during predive training. Several possible mechanisms responsible for this effect include: firstly, lean body conformation which reduces total N_2 uptake and tissue saturation and attenuates release of N_2 into the blood during decompression; secondly, increased capillarization of muscles by training to promote efficient transfer of gas into the blood by decreasing inert gas tension within individual capillaries, and thereby reducing extravascular gas nucleation and coalescence; thirdly, the improved cardiovascular function (e.g., increased myocardial contractility, stroke volume, cardiac output) may assist in improving N_2 elimination.

The inability of swimming exercise to affect decompression tolerance may have been due to the inefficiency of the exercise regime. There is no known reason for the failure of the cold acclimatized mice to affect decompression tolerance. Previously it has been reported that intermittent exposure to severe cold has a beneficial decompression effect. One possible reason for the negative effect of hypoxia acclimatized mice on alleviation of decompression sickness may have been the deleterious effects polycythemia and the accompanying increased blood viscosity that was produced by the predive treatment. This circulatory change could promote gas nucleation and bubble formation.

Experiments designed to determine endocrine and morphological responses by exposure to high altitude (hypoxia) are being conducted in mice. Plasma profiles of reproductive hormones (LH, FSH, Prolactin, Testosterone) and corticosterone are being determined in mice exposed continuously either to 760 Torr (sea level control), 379 Torr (18,000 ft), or 321 Torr (22,000 ft) either prior to hypoxia or after 1, 3, 7, 14 or 28 days of treatment. Similar hormone analyses are being made on mice acutely exposed to either 632, 523, 429, 349 or 282 Torr (simulated altitudes from 5,000 to 25,000 ft). Also, an investigation has been initiated studying the effects of age on altitude tolerance, using survival and plasma enzyme profile threshholds to measure tolerance.

Publications:

Altland, P. D. and Rattner, B. A.: Effects of nicotine and carbon monoxide on tissues and systemic changes in rats. Environmental Research. In press.

Rattner, B. A., Gruenau, S. P. and Altland, P. D.: Cross-adaptive effects of cold, hypoxia, or physical training on decompression sickness. J. Appl. Physiol.: Resp., Environ., Exer. Physiol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29015-08-LCP

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Digital computer facilities for LCP and LMB

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William H. Jennings, Jr.	Research Physicist	A LCP
OTHER:	Arthur R. Schultz, Jr.	Electronic Engineer	CR, CSL
	John I. Powell	Electronic Engineer	CR, CSL
	Eugene O'Bryan	Electronic Engineer	CR, CSL
	Marie A. Chang	Mathematician	CR, CSL
	Ronald Fico	Electronic Engineer	CR, CSL

COOPERATING UNITS (if any)

Computer Systems Laboratory, DCRT

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Membrane Biophysics

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The laboratory computer facility serving LCP and LMB is being converted from the existing star configuration to a three level hierarchical system. The direct memory linkage between the store and forward processor and the data processor is installed and under test. The prototype dedicated micro computer has been in routine operation in the laboratory for six months.

PHS-6040
(Rev. 10-76)

Project Description:

This computer facility has served the laboratories of Molecular Biology and Chemical Physics for the past ten years. In cooperation with the Computer Systems Laboratory, DCRT we are beginning to replace the obsolete components of this system. This transition will cause minimum disruption of laboratory work because both the new and the old systems will operate in parallel. The existing centralized or star architecture is being converted to a three level hierarchical configuration. This arrangement consists of (1) a dedicated micro-computer at each instrument to perform the real-time data acquisition and control functions, (2) a serial link to a store and forward communications processor and (3) a direct memory channel linking the communications processor to the existing central processor (a Honeywell 516 machine). When all instruments have been converted to the new configuration, a graceful replacement of the Honeywell device is possible since then only one link connects it with the rest of the system.

Objectives:

Development of an integrated hardware-software system to support data acquisition from research instruments and the processing, plotting and archiving of that data.

Findings:

The major effort this year has been the design and installation of a high speed link between a DEC LSI-11 communications processor and the Honeywell 516. This link uses direct memory hardware to transfer data at speeds of about 250Kbits/sec from the memory of one device to that of the other. Four main programs implement a block transfer communications protocol that employs a control block with column and cyclic redundancy error checking. On the communications processor an executive handles requests for service from serial lines to the instrument micro-computers and queue requests for file transfer via the high speed link. The file transfer process is executed by a program that implements the block transfer protocol and returns error information to the executive. On the Honeywell machine a similar file transfer programs implements the block transfer protocol and runs under the real-time operating system OLERT in parallel with data acquisition and processing programs. The direct memory channel is accessed via a device driver designed to run as part of the OLERT system software. On the Honeywell 516 a normalization program translates integer data files into scaled floating point files suitable for processing. This communications link has been operational for five months, undergone several revisions and is now under test for throughput and error recovery.

In anticipation of the availability of a DEC 11/70 machine from LMB, DEC hardware and software have been evaluated with the assistance of CSL staff and the details of replacing the Honeywell 516 have been specified. Development of the replacement system will be carried out principally on an 11/70 system purchased by CSL, DCRT for prototype development. This arrangement will enable us to have most of the new software available in a tested form when the Honeywell machine is removed.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AM-29016-04-LCP

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Macromolecular assembly reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James Hofrichter	Research Chemist	A:LCP
COLLABORATORS:	William A. Eaton	Medical Director	A:LCP
	Frank A. Ferrone	Staff Fellow	A:LCP
	Helen R. Sunshine	Staff Fellow	A:LCP

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Physics

SECTION
Spectroscopy and Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Laser photolysis of samples liganded with carbon monoxide is being used to rapidly form deoxyhemoglobin S in order to study the kinetics of the polymerization reaction. The time resolution of these kinetic experiments is being extended by the construction of a transient laser spectrometer which will permit accurate absorption spectra to be obtained in times as short as 10 nanoseconds after photolysis.

PHS-6040
(Rev. 10-76)

Methods Employed:

Both theoretical and experimental techniques are being used to study assembly reactions. The intensity of light scattered by assembling protein solutions is being studied as a function of angle and protein concentration in order to obtain information on the molecular weights and virial coefficients of the proteins and non-assembling controls. The intensity of scattered light is also being used as a probe to examine the kinetics of the polymerization and/or assembly reactions.

Conventional sedimentation techniques which measure the solubility of the hemoglobin S with respect to the polymer phase and turbidimetric measurements of the rate of appearance of the polymer have been supplemented by linear dichroism measurements in an investigation of the polymerization of partially liganded hemoglobin S solutions. The linear dichroism has the advantage of observing only the hemoglobin which is spontaneously aligned and thus is capable of spectrophotometrically monitoring the composition of the polymer phase without interference from the protein in the solution phase.

Both light scattering and linear dichroism measurements have also been utilized in kinetic experiments in which polymerization is induced by laser photolysis of hemoglobin S saturated with carbon monoxide. In experiments to date, an argon-ion laser has been used as a photolysis source. The capabilities of the photolysis technique are now being significantly expanded by the design and acquisition of a pulsed laser spectrometer. The basic components of the spectrometer are an excitation source which initiates the process (photolysis of HbCO or orientation of Hb S aggregates), a probe source for monitoring the process, a detection system, which permits the measurement of the wavelength dependence of an optical property in a finite time interval or of the time dependence of an optical property in a finite wavelength interval, and finally a data processing system.

As an excitation source we will use a Q-switched, flash lamp pumped Nd:YAG laser. The fundamental frequency of this source if 1064 nm, very close to the absolute absorption minimum of a deoxyhemoglobin solution. The high peak intensity of Nd:YAG (10^8 watts) permits efficient optical mixing to generate intense harmonics at 532 nm, 355 nm, and 266 nm, which can then be used directly for excitation. The fundamental and harmonics may also be used to pump dye lasers, providing continuous tunability from the ultraviolet to the near infrared. Tunability is necessary in the excitation source in order to maximize the quantum yield of photolysis and the orientation of aggregates and to minimize the heating in both classes of experiments.

Two alternative methods will be used to probe the progress of the reaction initiated by the excitation source. The first is the recording of transient spectra at predetermined times after the flash. In this experiment an extremely short flash of light having a wide frequency bandwidth is passed through the excited sample. The pulse of light is then dispersed by a monochrometer and the intensity profile recorded at all wavelengths simultaneously.

The comparison of the intensity profile at this point with an intensity profile measured without excitation gives an accurate difference spectrum. A very high source intensity is required for this experiment in order to permit the acquisition of high quality spectroscopic information in a single flash. To provide the high intensity a Nd:YAG laser of lower total power will be used to produce a third harmonic beam at 355 nm, which will, in turn, be used to excite fluorescence from dyes having lifetimes for emission of the order of 1 ns. The resulting broad-band dye fluorescence provides a noise-free continuum for measuring spectra. In addition, the width and range of the emission can be selected for each experiment by selecting appropriate dyes. The duration of the pulse is slightly longer than the exciting laser pulse due to the finite lifetime of the dye fluorescence, but practical pulse durations of 5-10 ns will be used.

The second method used to monitor the reaction will be to measure the time course of the kinetics at a fixed wavelength. This experiment requires a monochromatic source of variable wavelength and a temporal duration which is longer than the kinetic process under investigation. For slow experiments, this requirement is met by DC xenon arc lamps. For faster processes, a source of higher intensity is required. Xenon flashlamps having durations of 1 μs to 1 ms produce several hundred times the source brightness of a DC lamp and will be used together with existing monochromators to provide single wavelength illumination. A desirable alternative, particularly for resolving small changes in photoselection experiments or very rapid transients, is to take advantage of a CW dye laser as the tuned probe source. The higher photon flux from the dye laser would both improve signal-to-noise and considerably simplify the design of the spectrometer.

For single wavelength kinetic studies, photomultipler tubes will be used together with two 50 mHz amplifiers and a dual-channel transient digitizer having 10 ns time resolution. For processing, the data will be rapidly transmitted from the transient digitizer to a small computer, such as the Hewlett-Packard 9825, which currently exists in the Laboratory.

For detection and processing of transient spectra a totally different kind of system is required. We will use a two-dimensional, image-intensified vidicon tube in conjunction with a spectrograph. This detector satisfies the criteria necessary to measure accurate transient spectra by recording the transmitted intensity from both the sample and blank over the desired wavelength range simultaneously. The two-dimensional resolution of this detector also provides the capability of measuring linear dichroism in split beam experiments by recording the two orthogonal polarizations simultaneously. For data reduction of the output from the vidicon we will use a microprocessor and display console specifically designed for computing spectra from the observed signals.

Major Findings:

I. Photolysis of Carbonmonoxyhemoglobin S

In the past year the photolysis experiments have proceeded through three phases:

A. Conditions under which polymerization could be induced by photolysis without the undesirable side effects of sample heating have been found and control experiments performed to accurately establish the magnitude of the temperature change. The samples required are extremely small, ~1-2 microns thick and 10^{-20} microns in diameter, necessitating the use of a microspectrophotometer to observe and measure the optical signals which are used to monitor the reaction. The very small volumes which are necessary to maintain temperature control made it nearly impossible to measure either turbidity or birefringence, the standard techniques by which we have observed polymerization. Fortunately, the scattering of light from the photolysis source itself provides a sensitive probe for the appearance of polymers, and the very high extinction coefficient of the Soret band provides a sensitive spectrophotometric measure of the fractional saturation of these small samples.

B. A quantitative investigation of the technique has been completed. Control experiments have been performed which examine several critical parameters of the experiments. First, the fractional saturation obtained in the presence of the laser light has been measured both as a function of power density and as a function of time after turning on the laser. These experiments show that it is possible to achieve greater than 97% desaturation within 1 millisecond. The results are readily modelled using known quantum yields for CO photolysis and recombination rate for CO with hemoglobin. Second, the temperature rise in the experiments has been measured by two independent techniques which yield consistent results. The temperature rises encountered in the laser beam are ypically 8-12°C under standard experimental conditions. The temperature rise is also consistent with detailed calculations of sample heating using the heat diffusion equation.

C. A systematic study of the photolysis-induced polymerization on completely desaturated samples is in progress. Using this technique we have been able to induce and observe polymerization over 1,000 times faster than was previously possible. The polymerization shows features which are qualitatively similar to those observed in slower experiments, viz., a delay period followed by rapid polymer formation. We have observed delays as short as 4 milliseconds. The delay is proportional to a very high power of the hemoglobin concentration (in our present experiments, 30th power) as observed in the slower experiments. The temperature dependence of the delay is also large and shows an apparent temperature dependence to the activation energy. At short times, however, the polymerization progress curve is considerably broadened, exhibiting only about 3rd order time dependence, compared to about 15th order dependence at longer times. The rates of photolysis-induced polymerization have also been measured on intact sickle cells, using light scattering as a

probe. We have found that partial photolysis of the intracellular hemoglobin can cause polymerization to occur within a few hundred milliseconds.

The rapid polymerization experiments provide three important pieces of information. First, the kinetic hypothesis which correlates the severity of sickle cell disease with the delay time requires that the intracellular hemoglobin polymerize in times short compared to capillary transit times, typically about 1 second. In contrast to a variety of reports in the literature, our photolysis experiments verify that this is indeed possible, and will provide quantitative data on rates of intracellular polymerization. Secondly, the ability which we now possess to measure polymerization kinetics over eight decades of time at a single temperature permits more stringent tests of the polymerization mechanism than have been possible to this point. For example, in fast experiments we should be able to kinetically distinguish the growth of polymers from their alignment, and thereby restrict possible mechanisms for the growth phase of the kinetics.

Significance to Biomedical Research and the Program of the Institute:

The studies of photolysis induced polymerization kinetics provide the only available technique for studying the polymerization reaction as it is actually initiated in vivo, by the removal of ligands on physiological time sides. The fast kinetics also show promise as a convenient screening technique for therapeutic inhibitors of hemoglobin S polymerization.

The physical studies of the polymer phase and polymerization kinetics are aimed at providing a better basic understanding of the physical nature of the polymer phase, and the self-assembly process by which it is formed.

Proposed Course of Project:

Laser photolysis experiments will be completed under conditions which produce complete desaturation. The techniques will then be exploited to examine the kinetic properties of single cells. A major objective of this study will be to determine whether photolysis induced polymerization can provide an efficient tool with which to screen potential therapeutic agents. The photolysis studies will also be used to examine kinetics in partially saturated solutions.

Modulated excitation experiments will be performed to examine the fractional saturation of the polymer phase as a function of solution fractional saturation. Presently available data from light scattering and birefringence will be analyzed to provide a sufficient model for the behavior of the polymer phase in melting and temperature perturbation experiments.

The construction and testing of the nanosecond spectrometer will assume high priority. Several straightforward experiments including measurement of rotational diffusion of hemoglobin photoselected by laser photolysis of carbon monoxide, standard flourescence lifetime experiments, and repetition of microsecond experiments on hemoglobin recombination kinetics will be used to test

the instrumentation as it is developed. The important technical problem of developing a catalog of fluorescent dyes and testing the maximum laser stimulated fluorescent intensity which can be derived from them will also be addressed. The feasibility of using very high laser fields to perturb the orientation of macromolecules will also be assessed by testing the maximum field strengths which can be sustained.

Publications:

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REPORTED IN PRESS - 1978

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Eaton, W.A. and Hofrichter, J.: Successes and failures of a simple nucleation theory for sickle hemoglobin gelation, ibid., (1978), pp. 443-457.

Hofrichter, J.: Ligand binding and the gelation of sickle cell hemoglobin. J. Mol. Biol., 128: 335-369 (1979).

Sunshine, H.R., Hofrichter, J. and Eaton, W.A.: Requirements for therapeutic inhibition of sickle hemoglobin gelation, Nature 275: 238-240 (1978).

ANNUAL REPORT
LABORATORY OF PHYSICAL BIOLOGY
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Muscle Contraction

X-ray diffraction. Since the X-ray diffraction pattern of muscle cells is sensitive to the degree of overlap of the myosin-containing and actin-containing filaments, and therefore on sarcomere length, a method of measuring this parameter was added to the Laboratory's X-ray diffraction setup. This was done by passing a laser beam through the specimen and recording the light diffraction pattern together with the X-ray diffraction pattern. The added experimental control made it possible to improve the quality of the data obtained from simplified muscle preparations. (Shapiro, Naylor)

The lateral spacing between the myosin-containing and actin-containing filaments in chemically "skinned" rabbit psoas muscle was found to decrease significantly when force was generated either by activation with calcium at a given sarcomere length, or by stretching the fiber in an EGTA solution until resting tension developed. This indicates that (1) cross bridges between the two types of muscle filaments develop radial as well as axial forces (see section below), and (2) resting tension in muscle cells may be an active force produced by cross bridges rather than a passive force resulting from strain of an elastic structure within the sarcomere. The intensity ratio of the two main equatorial reflections, I_{10}/I_{11}, which gives information about the position of the myosin molecules in the filament lattice, decreased by about 25% when force was generated by calcium activation of rabbit psoas preparations at 4°C. This is a considerably smaller change than that seen in intact frog fibers at that temperature, possibly because of the smaller force produced by rabbit fibers. (Shapiro, Tawada, Podolsky) This ratio was also studied as a function of the degree of activation in intact frog fibers. Activation was controlled by means of caffeine, which releases calcium from the sarcoplasmic reticulum when the temperature of the cell is quickly lowered. The intensity ratio I_{11}/I_{10} varied gradually with the isometric force level. A two state model in which myosin molecules are either in a resting or an attached state, and in which force is proportional to the fraction of the molecules in the attached state, was used to calculate the intensities of the individual reflections. The data for I_{10} deviated from this model in a way that suggests that formation of the first few cross bridges decreases the regularity of the remaining unattached myosin molecules. (Yu)

Synchrotron radiation. Diffraction experiments with muscle cells were made at the Stanford Synchrotron Radiation Laboratory. The aim was to record the equatorial intensities, I_{10} and I_{11}, and the higher order reflections, I_{20}, I_{21}, I_{30}, etc. in quickly shortening muscle cells, using a stroboscopic method to accumulate counts. The beam at Stanford was "up" during only a small fraction of the experimental period, so the results were scanty. However, the effort was successful in the sense that high quality diffraction data were

collected 10 to 100 times faster than with the rotating anode X-ray generator at NIH, and it was shown that kinetic experiments with muscle cells would be feasible if the synchrotron were operated in a mode dedicated to radiation production. (Naylor, Yu, Crist, Podolsky)

Radial cross bridge force. The geometry of the way the myosin molecule is believed to interact with an actin filament to produce force in the axial direction is such that a component is present in the radial as well as the axial direction, which tends to decrease the spacing between the myofilaments. The expected magnitude of this effect has been calculated. The effect is too small to detect in an intact muscle fiber where the osmotic force is much greater than the radial cross bridge force, but the computations suggest that in a skinned muscle fiber, where osmotic constraints are eliminated, the predicted filament spacing change upon activation should be quite measurable (see section on X-ray diffraction). Thus radial cross bridge forces are not negligible in comparison to van der Waals and electrostatic forces. (Schoenberg)

Rate limiting step in the cross bridge cycle. The steady shortening rate of muscle cells is thought to be determined by the rates of "making" and "breaking" cross bridges, which, in fibers, are reflected in the transient response to step changes in load. As expected, the time course of these transients depends on parameters that affect acto-myosin interaction in solution (ionic strength, nucleotide concentration, etc.). However, the steady motion was found to be insensitive to these parameters. This indicates that the rate limiting step in the cross bridge cycle may be an intramolecular transition in the myosin molecule that occurs in the detached state (which has been characterized in solution by E. Eisenberg, NHLBI) rather than the making and breaking of cross bridges *per se*. (Tawada, Podolsky)

The activation mechanism. Analysis of the regulation of intracellular calcium movement in muscle cells was extended using techniques previously developed for simultaneous measurement of ^{45}Ca movement and isometric force in skinned frog fibers. Previous work has shown that stimulation of ^{45}Ca efflux by "depolarization" is highly calcium-dependent. The results of studies designed to characterize the calcium-dependence suggest that stimulated calcium efflux from the sarcoplasmic reticulum utilizes specific membrane carriers or channels that are regulated by calcium itself and does not represent a simple increase in permeability. The evidence is as follows: The presence of a calcium chelator inhibited stimulation of ^{45}Ca efflux by reduction of free magnesium (20 M to 4 M Mg^{++}) or by caffeine. Inhibition was complete for magnesium reduction and partial for caffeine, showing that caffeine can initiate stimulation at a site that is either inaccessible to the chelator or calcium-insensitive. The residual efflux in caffeine had a delayed maximum, and the peak rates were reduced slightly at increased chelator concentration, suggesting that the chelator has only a limited ability to control calcium-sensitive sites when calcium efflux is very large. Stimulation of ^{45}Ca efflux by 5 mM caffeine was reduced at higher free magnesium levels (0.7 mM Mg^{++}). The presence of

5 mM chelator was completely inhibitory at this magnesium, showing that 1) calcium-dependence is a property of stimulated calcium efflux at magnesium levels in the physiological range; 2) magnesium is a direct inhibitor of efflux stimulation by caffeine; 3) the effects of magnesium and chelation are synergistic. (Stephenson)

Techniques necessary for quantitative studies of ^{45} uptake and distribution of permeant ions in skinned frog fibers were further developed. The total solvent volume of fiber segments was estimated from ^{14}C-antipyrine uptake, measured with a newly devised method that minimized extraneous tracer. Total protein content was estimated with a new micromethod recently described in the literature. Measured on the same segment, solvent volumes (about 20-50 nl) correlated with protein content (about 1-5 μg). (Stephenson)

Cell Membranes

Phase transitions in lipid films. Studies of the formation of bimolecular lecithin surface films on water have been extended to other phospholipids, and to mixtures of dimyristoyl lecithin, (DML) and dioleoyl lecithin (DOL). In addition to various lecithins, sphingomyelin and phosphatidyl ethanolamine have been observed to form surface bilayers. Thus the major phospholipid components of cell membranes spontaneously form surface bilayers. The formation of the surface bilayers is temperature-dependent; it is stable over a limited temperature interval of about 5-10°, and the temperature is a characteristic of the particular phospholipid. When the temperature is raised above this interval, the surface bilayer undergoes a continuous transformation to a simple monomolecular state; thus the surface bilayer and monolayer coexist over a range of temperatures. The surface transformation from surface bilayer to monolayer appears to be a higher order phase transition since it does not occur at a unique temperature. (Gershfeld)

Disaturated lecithin analysis in plasma, and atherosclerosis. The finding of a strong correlation between the disaturated lecithin titer of plasma and the presence of atherosclerosis reported last year, has stimulated a search for an improved analytical technique suitable for clinical assays of these lipids. A number of techniques have been tried, among which mass spectrometry has proven difficult to establish quantitation. However, gas liquid chromatography has now been appreciably improved by the use of capillary columns. All disaturated derivatives of plasma lecithins are quantitatively separated using a 10 meter OV101 capillary column. The general procedure for quantitative analysis of disaturated lecithins is: a) extraction of lipids from plasma, using in succession 2-propanol and chloroform, b) thin layer chromatography of the extracted lipids to isolate lecithins, c) acetylation of the lecithins to diglyceride acetates, d) ozonolysis of the diglyceride acetates (to remove all unsaturated fatty acids, and e) gas chromatography of the residue. Quantitation is assured by including as an internal standard in (b) any of the lecithins with the following fatty acid composition: 14:0/14:0, 15:0/15:0 or 19:0/19:0. (Gershfeld)

Red cell membrane and cholesterol. The transport of lipids to and from the body tissues by plasma lipoproteins is an important part of whole body metabolism. The molecular level details of the transfer process are not well understood, although it is generally accepted that the transfer requires contact between lipoprotein molecules and tissue surfaces. Cholesterol exchange between lipoproteins and mammalian erythrocytes is a particularly simple prototype of such lipid transfer processes, since it does not appear to involve enzymes or energy sources, and the cholesterol of the erythrocyte is contained entirely within its plasma membrane. Cholesterol exchange between human erythrocytes and three of the density fractions of plasma lipoproteins, namely low density lipoprotein and the major sub-classes of high density lipoprotein, HDL_2 and HDL_3, has been studied. For this work, native erythrocyte cholesterol was partially replaced by C^{14}-labelled cholesterol. With all three lipoproteins, and at several temperatures, the exchange rates increased with lipoprotein concentration until a concentration was reached beyond which the rate remained constant. This limiting exchange rate was the same for the various lipoprotein species, and their mixtures. The simplest explanation of these findings is that at low lipoprotein concentrations the transfer rate is controlled by the frequency of collisions of lipoprotein molecules with the cell surface, while the rate becomes limited at higher lipoprotein concentrations by a slow process within the cell membrane, possibly the diffusion of cholesterol. The cholesterol exchange between erythrocytes and lipoproteins at the concentrations of normal human serum would be predicted to occur at the limiting exchange rate. (Gottlieb)

Molecular Weight by Radiation Inactivation

Radiation inactivation of enzymatic activity or hormone binding can be analyzed by target theory to yield the molecular weight of the functional unit. Using this approach, several polyenzymes and membrane systems are being studied. Preliminary results indicate that all the enzymatic activities in a single protein show the same radiation sensitivity, and therefore the same functional unit size. Hormone binding by membrane receptors shows unique radiation sensitivity. The glucagon receptor and nucleotide regulatory units exist as multimers in the membrane, but dissociate in the process of combining with the enzyme. Insulin binding has been found to be controlled by a large inhibitor. Thyroxin binding by chromatin is greatly dependent on the preparation methods used for chromatin isolation. (Kempner)

Morphogenesis

Insect metamorphosis. Calliphorin is a major storage protein in insects. Injection of labeled calliphorin into mature blowfly larvae revealed that radioactivity derived from calliphorin is present to a varying degree in all the adult organs examined. The protein is actually a family of 6-7 closely related hexameric protein species, each of which is itself composed or 6 monomers. Separation and isolation of either individual hexamers or

monomers, is unexpectedly difficult. Indirect evidence indicates that association of monomers--a function of both salt concentration and pH--into oligomers is not random, and that each hexamer is probably homogeneous with respect to monomers. Some 15 percent of all tyrosine residues appear to be exposed to the solvent in hexameric calliphorin; this value increases to 25 percent under conditions of reversible dissociation into monomers, and by a further 25 percent under irreversible conditions. Only monomers and/or hexamers are observed in the ultra-centrifuge, but by using dimethyl suberimidate or tetranitromethane as intermediate "trapping" reagents, the entire range of possible oligomers was demonstrated electrophoretically. A variety of chemical modification reactions indicated that increasing the negative charge, or decreasing the positive charge of the monomers prevents their association, and that tyrosine and lysine residues are important in the reversible association of these subunits. (Levenbook, Bauer, Zomer)

Red cell sickling. Deoxygenated sickled human erythrocytes orient in a magnetic field. The high-spin paramagnetic moment of heme is believed to be the source of the orientation effect. Because paramagnetism (a consequence of free unpaired electrons) is usually 1000-fold greater in magnitude than diamagnetism (an inductive effect due to electron orbits in an external magnetic field), the latter is generally considered to be negligible when both are present. However, oxygenated diamagnetic sickled deer cells also orient in a magnetic field. This observation, along with the working model for human polymerized deoxygenated hemoglobin S, implicates heme diamagnetism in the orientation effect. Calculations have shown that in human sickle cells both paramagnetism and diamagnetism contribute to the orientation while diamagnetism alone accounts for the deer cell orientation. (Murayama, Adams)

There is evidence that orally administered urea may serve as a prophylactic for the hand-foot syndrome associated with sickle cell disease. A clinical test has been carried out in West Africa under the supervision of Dr. Roger Lewis. The results are as follows:

Groups	Cases	Average Age	History of Previous Attacks of Hand-foot Syndrome	Average Observation Period (months)	Number of Attacks
Control	10	2 6/12	9	14	7
Low urea	8	2 7/12	6	8	5
High urea	8	2 7/12	7	11	0

(Murayama)

Bioluminescence

In pursuance of the overall objective of understanding how neural oscillators control efferent processes and interact with each other, efforts have been made to deduce, from the temporal pattern of light emission, how the endogenous pacemaker cycles and how it is influenced by visual input. Statistical tests for Gaussian distribution of free run interflashes of the male of the firefly Pteroptyx cribellata are consistent with the thesis that the photic output is heterogeneous--that is, that it includes excitation from other endogenous and exogenous sources in addition to the primary pacemaker rhythm. Analysis of records in which either main rhythm flashes are skipped or adventitious extra luminescences occur strongly suggests that a delay of 50-75 msec per 1000 msec timing cycle is incurred in dispatching a motor message to the lantern. (Buck)

High Resolution Electron Microscopy

A new facility is being established for structural studies on biological macromolecules and macromolecular assemblies by high resolution electron microscopy reinforced by optical diffraction and computer image processing. This facility is partially operational and work has been started on several systems: the human erythrocyte membrane, microcrystals of human immunoglobin molecules, and a filamentous plant virus. (Steven, Gottlieb, Navia)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 27000-17 LPB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Mechanism of Muscular Contraction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Podolsky	Chief, LPB	LPB NIAMDD
OTHER:	L. C. Yu	Research Physiologist	LPB NIAMDD
	E. W. Stephenson	Research Biologist	LPB NIAMDD
	G. R. S. Naylor	Visiting Fellow	LPB NIAMDD
	S. Matsubara	Visiting Associate	LPB NIAMDD
	P. J. Shapiro	Staff Fellow	LPB NIAMDD
	K. Tawada	Visiting Scientist	LPB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Cellular Physics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.25	5.25	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Muscle cells are activated when calcium is released from the sarcoplasmic reticulum into the myofilament space. The processes involved in this intracellular calcium movement have been studied by measuring isometric force and ^{45}Ca fluxes in mechanically skinned fibers from frog muscle. The structural changes associated with the transition of muscle cells from one physiological state to another have been examined by X-ray diffraction techniques, using radiation from conventional rotating anode generators and synchrotrons in conjunction with electronic, position sensitive, X-ray detectors. The rate constants associated with the turnover of actomyosin cross-bridges have been studied by analyzing the contraction kinetics of skinned muscle fibers under controlled chemical conditions.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.

Methods Employed:

1. Analysis of the motion and the X-ray diffraction pattern of both intact muscle fibers and "skinned" fiber segments under chemically controlled conditions.
2. Study of the electrical and chemical parameters involved in the activation of various types of muscle fibers.
3. Measurement of ionic fluxes across the internal membranes of skinned muscle fibers under various conditions.

Major Findings:

1. The steady shortening rate of muscle cells is thought to be determined by the rates of "making" and "breaking" cross bridges, which, in fibers, are reflected in the transient response to step changes in load. Although the time course of these transients depends on parameters that affect actomyosin interaction in solution (ionic strength, nucleotide concentration, etc.), the steady motion was found to be insensitive to these parameters. This indicates that the rate limiting step in the cross bridge cycle may be an intramolecular transition in the myosin molecule that occurs in the detached state rather than the making and breaking of cross bridges *per se*.
2. The intensities of the two strong equatorial X-ray reflections from frog muscle fibers, *10* and *11*, were measured at various levels of activation and compared with a model in which myosin molecules are either in a resting or an attached state, and in which force is proportional to the fraction of the molecules in the attached state. I_{10} deviated from this model in a way that indicates that formation of the first few cross bridges decreases the regularity of the remaining unattached myosin molecules.
3. The lateral spacing between the myosin-containing and actin-containing filaments in chemically "skinned" rabbit psoas muscle was found to decrease significantly when force was generated either by activation with calcium at a given sarcomere length, or by stretching the fiber in an EGTA solution until resting tension developed. This indicates that (1) cross bridges between the two types of muscle filaments develop radial as well as axial forces and (2) resting tension in muscle cells may be an active force produced by cross bridges rather than a passive force resulting from strain of an elastic structure within the sarcomere.
4. Analysis of the regulation of intracellular calcium movement in muscle cells was extended using techniques previously developed for simultaneous measurement of ^{45}Ca movement and isometric force in skinned frog

fibers. Studies of the calcium-dependence of the calcium efflux from the sarcoplasmic reticulum suggest that stimulated calcium efflux utilizes specific membrane carriers or channels that are regulated by calcium itself and does not represent a simple increase in permeability.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the molecular mechanism of muscular contraction together with the chemistry of the activation process, can be useful in the rational handling of neuromuscular and cardiovascular disease.

Proposed Course of Project:

1. The influence of various agents (pCa, pH, ionic strength, metal ions, ATP) on the ability of muscle fibers to develop force and to shorten will be examined directly in skinned fiber segments.
2. The influence of these same agents on myofilament spacing and the cross-bridge configuration will be examined in skinned fibers by X-ray diffraction.
3. The effect of motion on the number of cross-bridges in intact, electrically-stimulated muscle fibers will be studied by X-ray diffraction.
4. Movement of calcium and magnesium between the myofilament space and the sarcoplasmic reticulum will be studied by tracer methods.

Publications:

Gulati, Jagdish and Podolsky, Richard J.: Contraction transients of skinned muscle fibers: effect of calcium and ionic strength. J. Gen. Physiol. 72: 701-715, 1978.

Yu, Leepo C., Hartt, Jacqueline E., and Podolsky, Richard J.: Equatorial X-ray intensities and isometric force levels in frog sartorius muscle. J. Mol. Biol. (in press).

Podolsky, Richard J.: The rate limiting step in muscle contraction. Basic Res. Cardiol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 27001-05 LPB

PERIOD COVERED October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Contractility and Excitability of Skeletal and Cardiac Muscle

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Mark Schoenberg Medical Officer LPB NIAMDD

COOPERATING UNITS (if any)

Dr. Jay B. Wells, Medical Neurology Branch, NINCDS

LAB/BRANCH

Laboratory of Physical Biology

SECTION

Section on Cellular Physics

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have explored the effects of cross-bridge geometry upon the contractile forces in muscle. We found that the radial component of the cross-bridge force should shrink the myofilament lattice about 2% upon activation of a skinned fiber. In addition, the influence of cellular geometry upon the electrical excitability of cardiac Purkinje fibers was also investigated.

PHS-6040
(Rev. 10-76)

Project Description:

Goal: To understand the molecular and structural basis of muscular contraction.

Methods and Major Findings:

1) Previously we developed appropriate equations for describing the way in which the three-dimensional orientation of muscle cross-bridges affects the axial force a muscle exerts. Recently we extended these calculations to describe the radial components of the cross-bridge force. Although the major radial forces in muscle have been thought to be electrostatic and dispersive, surprisingly the predicted magnitude of the radial component of the cross-bridge force was not negligible. Experiments are currently underway here and in at least one university laboratory to experimentally confirm this prediction.

2) Resting muscle has been described as having a viscosity which appears to disappear upon activation. Preliminary experiments done this fiscal year suggest that this is not a true viscosity but more likely a short-range elasticity having a rapid time constant. This suggests that the "apparent viscosity" of resting muscle may be related to the behavior of muscle cross-bridges, and not to the viscosity of the myoplasm between the muscle filaments.

3) Our modelling of the active cardiac Purkinje fiber enabled us to theoretically study the effect of the Purkinje fiber clefts (deep intercellular spaces) upon excitability of that tissue. We found that although the presence of the clefts increased the charge threshold for excitation, it did so uniformly for both short and long duration stimuli. This implies that during excitation the membrane in the intercellular clefts behaves similarly to that on the surface membrane and does not act as either a source or sink for excitation currents.

Significance to Biomedical Research and the Program of the Institute:

Experiments on the mechanism of muscular contraction are especially significant in that firstly they contribute to a sound understanding of how normal muscle works (which is vital to the study of diseased muscle) and secondly, many of the same mechanisms responsible for muscle contraction appear to be common to virtually all other cells.

Future Course:

It will be important this coming fiscal year to further investigate the nature of the short range elasticity in resting muscle. We hope to discover whether this phenomenon exists in a "skinned" muscle fiber. If so, this will offer the opportunity to study the elasticity under conditions where the internal milieu of the cell may be varied.

Publications:

Schoenberg, Mark and Fozzard, Harry A.: The influence of intercellular clefts on the electrical properties of sheep cardiac Purkinje fibers. Biophys. J. 25: 217-234, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-27002-16 LPB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Sense of rhythm

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John B. Buck Research Physiologist LPB NIAMDD

OTHER: Mrs. Elisabeth M. Buck, Guest Worker; Mr. Charles H. Hanna

COOPERATING UNITS (if any)

Dr. Lesley Ballantyne, Riverina College of Adv. Ed., Wagga Wagga, Australia; Dr. Maria Neuwirth, LEP/NIAMDD; Mr. George Atta, DCRT

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Comparative Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2-1/4	1	1-1/4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The two manuscripts referred to in last year's report are still undergoing improvement. In particular the statistics of free run flashing has been re-examined, leading to indications that response latencies are heterogeneous and that the process that dispatches the motor message adds to the endogenous timing cycle.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

In pursuance of the overall objective of understanding how neural oscillators control efferent processes and interact with each other, we have been trying to deduce, from the temporal pattern of light emission, how the endogenous pacemaker cycles and how it is influenced by visual input.

Major Findings:

Results of the Kolmogorow-Smirnov and chi^2 tests for Gaussian distribution of free run interflashes of the male of the firefly Pteroptyx cribellata, as applied by Mr. Atta, are consistent with the thesis that the photic output is heterogeneous -- that is, that it includes excitation from other endogenous and exogenous sources in addition to the primary pacemaker rhythm. Analysis of records in which either main rhythm flashes are skipped or adventitious extra luminescences occur strongly suggests that a delay of 50-75 msec per 1000 msec timing cycle is incurred in dispatching a motor message to the lantern.

Mrs. Buck completed behavioral study of Luciola aphrogeneia, a firefly species remarkable because of living on dead coral cliffs (New Guinea) at the very verge of the sea (in press).

Mr. Hanna has collaborated in two studies in rat hepatocytes. With Dr. Ann E. Kaplan and associates (NCI) he helped demonstrate that cells transformed by nitrosomethylurea differ in a member of ultrastructural details from control cells. With Dr. Jean Hickman and associates (LBM/NIAMDD) he assisted in showing that polystyrene latex beads are phagotized singly, that each bead is completely enclosed by membrane and that the membrane thus acquired is inside/outside reversed. Attention is called to a nice mutual cooperation that has developed between our EM facility and that of NICHD (Dr. J. Maizel).

Significance to NIAMDD Research:

Insofar as the Institute supports attempts to broaden our understanding of basic biological processes it is hoped that our work is appropriate.

Proposed Course of Project:

It is proposed to complete the analysis of endogenous pacemaker mechanisms in synchronously flashing fireflies. During this coming year Dr. Neuwirth and Mr. Hanna will investigate lantern ultrastructure and cytochemistry.

Publications:

Ballantyne, Lesley A. and Elisabeth Buck: Taxonomy and behavior of Luciola (Luciola) aphrogeneia, a new surf firefly from Papua New Guinea. Trans. Amer. Ent. Soc. 105: (in press).

REPORTED IN PRESS - 1978

Buck, John: Functions and Evolutions of Bioluminescence. (Chapter) In Herring, P. J. (Ed.): Bioluminescence In Progress. New York, Academic Press. 1978. pp. 419-460.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 27003-10 LPB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Dynamic Properties of Cell Membranes and Related Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Norman L. Gershfeld Research Chemist LPB NIAMDD

COOPERATING UNITS (if any)
S. Razin, Department of Clinical Microbiology, Hadassah Medical School, Jerusalem, Israel

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Comparative Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major membrane phospholipids--lecithin, sphingomyelin, and phosphatidyl ethanolamine--have been shown to form spontaneously single bilayers at the water surface. Mixtures of these lipids will also form surface bilayers, but at temperatures which are intermediate between the temperatures at which the pure compounds form surface bilayers. The surface bilayers form only when in the presence of bulk lipid. The process of formation of the surface bilayers parallels the formation of membrane bilayers in that for both systems the saturated fatty acid composition increases when temperatures are elevated. The significance of this correlation as a possible mechanism for membrane biogenesis is being tested by studying the growth characteristics of the temperature sensitive bacillus stearothermophilus.

PHS-6040
(Rev. 10-76)

Project Description:

Major Findings:

I. Phase transitions in lipid films.

Studies of the formation of bimolecular lecithin surface films on water, first reported last year, have been extended to other phospholipids, and to mixtures of dimyristoyl lecithin, (DML) and dioleoyl lecithin (DOL). In addition to various lecithins sphingomyelin and phosphatidyl ethanolamine have been observed to form surface bilayers. Thus the major phospholipid components of cell membranes spontaneously form surface bilayers. The formation of the surface bilayers is temperature-dependent; it is stable over a limited temperature interval of about 5-10°, and the temperature is a characteristic of the particular phospholipid. Increasing the temperature above this interval the surface bilayer undergoes a continuous transformation to a simple monomolecular state; thus the surface bilayer and monolayer coexist over a range of temperatures. The surface transformation from surface bilayer to monolayer appears to be a higher order phase transition since it does not occur at a unique temperature.

When two lecithin components are present, the formation of surface bilayer occurs at a temperature which is intermediate between the respective gel-liquid crystal transition temperatures (T_c) of the pure components. Thus for equimolar mixed dispersions of DML and DOL surface bilayers form at about 10°C, an intermediate temperature between T_c for pure DML (23.5°C) and for pure DOL (-20°C). Moreover the temperature range is slightly broadened to about 10-20°.

II. Disaturated lecithin analysis in plasma, and atherosclerosis.

The finding of a strong correlation between the disaturated lecithin titer of plasma and the presence of atherosclerosis reported last year, has stimulated a search for an improved analytical technique suitable for clinical assays of these lipids. A number of techniques have been tried, among which mass spectrometry has proven difficult to establish quantitation. However, gas liquid chromatography has now been appreciably improved by the use of capillary columns. All disaturated derivatives of plasma lecithins are quantitatively separated using a 10 meter OV101 capillary column. The general procedure for quantitative analysis of disaturated lecithins is: a) extraction of lipids from plasma, using in succession 2-propanol and chloroform, b) thin layer chromatography of the extracted lipids to isolate lecithins, c) acetylation of the lecithins to diglyceride acetates, d) ozonolysis of the diglyceride acetates (to remove all unsaturated fatty acids, and e) gas chromatography of the

residue. Quantitation is assured by including as an internal standard in (b) any of the lecithins with the following fatty acid composition: 14:0/14:0,15:0/15:0 or 19:0/19:0.

Future course of study:

The general significance of surface bilayer formation in phospholipids will be examined by studying the lipids of bacillis stearothermophilus whose membrane lipids can be varied simply by changing the growth temperature. In addition the analysis of disaturated lecithins in human plasma will be extended to a wider population to include additional atherosclerosis-associated variables such as smoking and diet.

Publications:

Gershfeld, N. L.: Selective phospholipid adsorption and atherosclerosis. Science 204: 506-508, 1979.

Gershfeld, N. L. and Tajima, K.: Spontaneous formation of lecithin bilayers at the air-water surface. Nature 279: 708-709, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 27004-09 LPB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Physical Chemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Melvin H. Gottlieb Research Chemist LPB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Comparative Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The exchange of cholesterol between human erythrocyte membranes and three species of plasma lipoproteins, low density lipoproteins and the two major fractions of high density lipoprotein, HDL_2 and HDL_3, was studied. With all three lipoproteins, and over the temperature range 5°C - 37°C, exchange rates first increased with lipoprotein concentration, and then reached a limiting value at higher lipoprotein concentrations. This limiting exchange rate was the same for all lipoprotein species, and their mixtures, and has an activation energy of about 9 kcal. It is believed that the limiting rate is due to a slow process occurring within the cell membrane, possibly the diffusion of cholesterol. The cholesterol exchange between erythrocytes and lipoproteins in normal human serum would be predicted to take place at the limiting exchange rate.

PHS-6040
(Rev. 10-76)

Project Description:

The transport of lipids to and from the body tissues by plasma lipoproteins is an important part of whole body metabolism. The molecular level details of the transfer process are not well understood, although it is generally accepted that the transfer requires contact between lipoprotein molecules and tissue surfaces. Cholesterol exchange between lipoproteins and mammalian erythrocytes is a particularly simple prototype of such lipid transfer processes, since it does not appear to involve enzymes or energy sources, and the cholesterol of the erythrocyte is contained entirely within its single, plasma, membrane. During this past year cholesterol exchange between human erythrocytes and three of the density fractions of plasma lipoproteins, namely low density lipoprotein and the major sub-classes of high density lipoprotein, HDL_2 and HDL_3, was studied. For this work, native erythrocyte cholesterol was partially replaced by C^{14}-labelled cholesterol by a method whose validation was described in last year's Report. With all three lipoproteins, and at several temperatures (5°C, 23°C and 37°C), the exchange rates increased with lipoprotein concentration until a concentration was reached beyond which the rate remained constant. This limiting exchange rate was the same for the various lipoprotein species, and their mixtures. While the magnitudes were somewhat dependent on the particular erythrocyte sample, typical values are .20% min^{-1} at 37°C, .08% min^{-1} at 23°C and .03% min^{-1} at 5°C. The simplest explanation of these findings is that at low lipoprotein concentrations the transfer rate is controlled by the frequency of collisions of lipoprotein molecules with the cell surface, while the rate becomes limited at higher lipoprotein concentrations by a slow process within the cell membrane. This latter process may possibly be the diffusion of cholesterol, although the temperature coefficient of the limiting exchange rate corresponds to an activation energy, 9 kcal., which is only about half of that for cholesterol diffusion in phospholipid bilayers. The cholesterol exchange between erythrocytes and lipoproteins at the concentrations of normal human serum would be predicted to occur at the limiting exchange rate.

In collaboration with Dr. A. Steven, work was started on the characization of the proteins which form two-dimensional arrays in certain erythrocyte ghost preparations. This work will be discussed in detail in Dr. Steven's report (Z01 AM 27009-01 LPB).

Publications:

Gottlieb, M. H.: A method for the increased extraction of cholesterol from human red blood cells by modified plasma lipoprotein. Prep. Biochem. 8: 463-469, 1978.

Gottlieb, M. H.: Validation of an in vitro method for incorporating labelled cholesterol into human erythrocytes. Prep. Biochem. 9: 247-259, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-27006-22 LPB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Developmental biochemistry of insect metamorphosis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. Levenbook	Research Chemist	LPB	NIAMDD
OTHER:	A. Bauer	Research Physiologist	LPB	NIAMDD
	E. Zomer	Visiting Fellow	LPB	NIAMDD

COOPERATING UNITS (if any)
None

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Comparative Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Physico-chemical characterization of the blow-fly storage protein calliphorin has been undertaken. The reversible monomeric-hexameric state of the protein has been studied as a function of pH and salt concentration, and intermediate oligomers "trapped" by use of cross-linking reagents or by chemical modification of specific protein groups. The conformational state of the protein has been examined by circular dichroism and difference spectroscopy. A variety of "state of the art" physical separation methods has been used in unsuccessful attempts to isolate pure, single calliphorin monomers and/or hexamers. Radioactive calliphorin was employed in preliminary experiments to determine the fate of the injected protein during metamorphosis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

(1) The role of tyrosine and lysine residues in the reversible association-dissociation of calliphorin oligomers.
(2) Identification of the types of pH sensitive forces involved in oligomer formation.
(3) The fate of calliphorin during its utilization *in vivo*.

Methods Employed:

Circular dichroism and ultra-violet spectroscopy; iso-electric focusing; a variety of gel electrophoretic techniques; electrometric titration; various chemical procedures for the study of protein structure.

Major Findings:

Pure calliphorin is actually a family of 6-7 closely related hexameric protein species, each of which is itself composed of 6 monomers. Separation and isolation of either individual hexamers or monomers, is proving to be unexpectedly difficult. Indirect evidence indicates that association of monomers -- a function of both salt concentration and pH -- into oligomers is not random, and that each hexamer is probably homogeneous with respect to monomers.

Some 15 percent of all tyrosine residues appear to be exposed to the solvent in hexameric calliphorin; this value increases to 25 percent under conditions of reversible dissociation into monomers, and by a further 25 percent under irreversible conditions. The increase in exposed tyrosine residues is accompanied by small, but progressive conformational changes in the protein. Only monomers and/or hexamers are observed in the ultra-centrifuge, but by using dimethyl suberimidate or tetranitromethane as intermediate "trapping" reagents, the entire range of possible oligomers was demonstrated electrophoretically. A variety of chemical modification reactions indicated that increasing the negative charge, or decreasing the positive charge of the monomers prevents their association, and that tyrosine and lysine residues are important in the reversible association of these subunits.

Preliminary experiments involving the injection of labeled calliphorin into mature blowfly larvae revealed that radioactivity derived from calliphorin is present to a varying degree in all the adult organs examined.

Significance to NIAMDD Research:

Insect metamorphosis is a programmed process under hormonal control which entails the decay and destruction of the larval cells concomitantly with the differentiation of the cells and structures that will give rise to the adult insect. The greater part of this process takes place within the span of a week. This telescoping of the aging and development process in the insect might be expected to bring forth features otherwise unobservable in the corresponding processes of man and mammals, which take place in a much more expanded time scale. In particular, the study of the fate of the principal soluble larval protein refers to the fate of an essential component of the developmental machinery and its products during aging and development.

Proposed Course of Projects:

(1) Isolation of individual calliphorin hexamers and monomers for chemical characterization and a study of their interrelationships.
(2) The fate of injected labeled calliphorin during metamorphosis of the blowfly.

Publications:

Levenbook, C.: Hemolymph volume during growth of Calliphora vicina larvae. Ann. Ent. Soc. Amer. (In Press).

Pau, R., Levenbook, L. and Bauer, A. C.: Inhibitory effect of β-ecdysone on protein synthesis by blowfly fat body in vitro. Experientia. (In Press).

REPORTED IN PRESS - 1978

Bauer, A. C., Chrambach, A. and Levenbook, L.: Molecular forms of four aldolases from larval and adult stages of the blowfly Phormia regina (Meig.). Insect Biochem. 8: 443-447, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-27007-17 LPB

PERIOD COVERED October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Magneto Optic Investigation into Human Red Cells (with HbS) "Sickling" and "Desickling"

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Murayama	Research Chemist	LPB-NIAMDD
OTHER:	A. Adams	Staff Fellow	LPB-NIAMDD

COOPERATING UNITS (if any) Dr. Oku Ampofo, Dr. Roger Lewis, Dr. M. Appah, Tetteh Quarsh Memorial Hospital and Ministry of Health, Mampong-Akwapim, Ghana; Dr. Robert M. Nalbandian and Prof. Ray Henry, Dept. of Physiology, Wayne State University, Detroit, Michigan.

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Comparative Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-3/4	1-3/4	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) Birefringence of human sickled red cells in physiological saline is lost when 10 - 20 mM carbamide is added.

(2) A two year double blind study (8 and 10 patients in experimental and control groups respectively) indicates that daily oral intake of one gram of urea per kilo body weight prevents onset of hand-foot syndrome (the initial sign of sickle disease crisis).

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

In 1964 Murayama demonstrated that deoxygenated sickled human erythrocytes orient in a magnetic field. The original report indicated the high-spin paramagnetic moment of heme as the source of the orientation effect. Because paramagnetism -- a consequence of free unpaired electrons -- is usually 1000-fold greater in magnitude than diamagnetism -- an ever-present inductive effect due to electron orbits in an external magnetic field, the latter is generally considered to be negligible when both are present. We have, however, found that oxygenated diamagnetic sickled deer cells also orient in a magnetic field. This observation, along with the working model for human polymerized deoxygenated hemoglobin S, implicates heme diamagnetism in the orientation effect. Calculations have shown that in human sickle cells both paramagnetism and diamagnetism contribute to the orientation while diamagnetism alone accounts for the deer cell orientation. Thus a 3 kGauss magnetic field will orient human cells while a 10 kGauss magnetic field is required for the deer cells.

In order to accurately quantify the directional magnetic parameters of the sickle cell and those of oxy- and deoxyhemoglobin we now plan to: (1) measure the magnetic susceptibility of ordered sickle cells both parallel and perpendicular to the sickle axis. (2) determine the magnetically induced birefringence of oxy- and deoxyhemoglobin solutions.

Major Findings:

In the laboratory the year has been mostly devoted to developing methods and perfecting the instrumentation, particularly to enable magnetic orientation and birefringence of red cells to be measured simultaneously. The surprising loss of birefringence in dilute urea (summary) is being investigated further. The clinical test of oral urea in West Africa under the supervision of Dr. Roger Lewis is summarized in the table below. Further cooperative clinical trials of the therapy in various parts of the world are continuing as opportunity offers.

ORAL PROPHYLACTIC UREA AND HAND-FOOT SYNDROME

GROUPS	CASES	AVERAGE AGE	HISTORY OF PREVIOUS ATTACKS	AVERAGE OBSERVATION PERIOD (MONTHS)	NUMBER OF ATTACKS
CONTROL	10	2 6/12	9	14	7
LOW UREA	8	2 7/12	6	8	5
HIGH UREA	8	2 7/12	7	11	0

Significance to NIAMDD Research:

Sickle cell anemia is a hereditary, hemolytic anemia -- an inborn error of metabolism; hence, exceedingly significant to the NIAMDD mission. The molecular basis for red cells (with Hb S) "sickling" is genetic interchange of a polar group, glutamyl with a hydrophobic residue, valyl at beta 6. This hydrophobic residue is so situated on the surface of the molecule that tetramer-tetramer interaction occurs by way of hydrophobic interactions; this has been confirmed by a large number of investigators. Murayama's theory has been tested at the bedside with success in Michigan as well as elsewhere (Santiago, Chile; Modevideo, Uraguay; Havana, Cuba; Mampong-Akwapim, Ghana; and so forth).

Proposed Course of Project:

The two main courses of the project are to investigate a co-factor of polymerization of Hb S and the role of zinc in sickle cell disease. Serum zinc will be assayed by the standard, atomic absorption spectrophotometry; assay method for the co-factor still needs to be developed.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM-27008-20 LPB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies of metabolic activity in microorganisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. S. Kempner	Physicist	LPB	NIAMDD
OTHER:	J. H. Miller	Chemist	LPB	NIAMDD

COOPERATING UNITS (if any)

Dr. Werner Schlegel, LNE-NIAMDD
Dr. Martin Rodbell, LNE-NIAMDD
Dr. Donald Gruol, LNE-NIAMDD
Dr. Virginia Patel, Univ. of Georgia
Dr. J. Z. Hearon, MB-NIAMDD
Dr. Joan Harmon, DB-NIAMDD

LAB/BRANCH

Laboratory of Physical Biology

SECTION

Comparative Physiology Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of metabolic pathways in axenic cultures of microorganisms are continuing. The relationships between biochemically sequential reactions are being examined.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

An understanding of the control of metabolic activity in unicellular organisms.

Methods Employed:

Culture of microorganisms in the presence of radioactive or other compounds; chemical and physical fractionation of the cellular materials by ultracentrifugation, chromatography and chemical extraction. Microscopic examination of cells by time-lapse video micrography. Electronic measurement of cell volume distribution and analysis in terms of cell growth and age. High energy radiation inactivation of enzymatically active cell extracts.

Major Findings:

The subcellular organization of biochemical processes continues to be the principal interest of this laboratory. This year the use of the radiation inactivation technique, which was re-introduced last year, has been expanded. In these studies, frozen or lyophilized preparations are irradiated with high energy electrons. Initial studies of the polyenzyme "arom" complex from Euglena gracilis indicated that two of the five enzymatic activities had the same radiation sensitivity. In collaboration with colleagues at the University of Georgia, "target analysis" of two other activities is now being conducted. Preliminary results indicate that the functional unit of all four enzymatic activities is the same, corresponding to the size of one chain in a two subunit protein molecule. Another polyenzyme system from bacteria, the aspartokinase-homoserine dehydrogenase complex, has been studied. Two different proteins are known to catalyze these reactions. One is a polyenzyme specific to the threonine-synthesizing pathway, while the other is specific for the methionine pathway. Both proteins catalyze both reactions; however, one is a tetramer, while the other is a dimer of different amino acid composition and is also immunologically unrelated. The radiation inactivation analysis revealed that for each species, the entire molecule was needed for enzymatic activity. Furthermore, in the case of the tetrameric enzyme, it was found that an ammonium sulfate precipitate of the enzyme revealed differing radiation sensitivities compared to purified samples. It appears that the precipitated form is dimeric, and that the structure is completely destroyed by ionizing radiation. Upon subsequent resuspension, only undamaged dimers can reconstitute a tetramer, which is the active unit. Mathematical analysis of this system was carried out by Dr. J. Z. Hearon.

In collaboration with Drs. Schlegel and Rodbell, radiation studies of the adenylate cyclase membrane system in rat liver were conducted. Target sizes for the glucagon-binding unit, the nucleotide regulatory structure

and the catalytically active cyclase unit were determined under various physiological conditions. These led to a considerable revision of the kinetic-structural models describing the various activities of the system. Most notably, it appears that the process of activation involves the disaggregation of an oligomeric structure.

Another membrane system involving insulin binding has been studied by Dr. Harmon. The radiation inactivation of the binding of insulin displayed remarkable and unexpected results: the absolute level of insulin bound increased continuously with radiation doses as high as ten million rads. At still higher doses, the binding capacity decreased, eventually showing a conventional exponential decay. This latter inactivation was shown to be due to destruction of the receptor site. Its size as determined by radiation analysis agrees well with the preliminary values obtained by conventional biochemical methods. The increase in activity described above has been interpreted by Dr. Harmon as the inactivation of a previously-unknown inhibitor of insulin binding. She has found the molecular weight to be 2-4 times larger than that of the receptor unit, and has further shown that the inhibitor acts by decreasing the affinity of the receptor site, not by decreasing the number of sites.

The binding of thyroxin to chromatin is also under study in collaboration with Dr. Gruol. Radiation inactivation of different chromatin preparations is giving new insight into the nature and size of the receptor site for T_3.

Publications:

Kempner, E. S. and Schlegel, W.: Size determination of enzymes by radiation inactivation. Anal. Biochem. 92: 2-10, Jan. 1978 (Invited Review).

Schlegel, W., Kempner, E. S. and Rodbell, M.: Activation of adenylate cyclase in hepatic membranes involves interactions of the catalytic unit with multimeric complexes of regulatory proteins. J. Biol. Chem. In press.

Kempner, E. S.: Metabolic compartments and their interactions. In "Cell Compartmentation and Metabolic Channeling". Eds., L. Nover, F. Lynen and K. Mothes. VEB G. Fischer Verlag, Jena and North Holland Publ. Co., Amsterdam (joint publishers), 1979. In press.

REPORTED IN PRESS - 1978

Kabuto, K., Imuta, M., Kempner, E. S. and Ziffer, H.: Product stereospecifity in the microbial reductions of hydroaromatic ketones. J. Org. Chem. 43: 2357-2361, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 27009-01 LPB

PERIOD COVERED October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Macromolecular Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alasdair C. Steven	Visiting Associate	LPB NIAMDD
OTHER:	Charles H. Hanna	Biol. Lab. Techn.	LPB NIAMDD

COOPERATING UNITS (if any)

Other collaborators on specific activities are identified below, in the accounts of these activities.

LAB/BRANCH

Laboratory of Physical Biology

SECTION

Section on Comparative Physiology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	1	0.75

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A facility is being established for structural studies on biological macromolecules and macromolecular assemblies by high resolution electron microscopy reinforced by optical diffraction and computer image processing. This facility is partially operational and work has been started on several systems: the human erythrocyte membrane, microcrystals of human immunoglobin molecules, and a filamentous plant virus.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To elucidate the processes of macromolecular assembly and function. Of particular interest, are studies on the structure and aggregation properties of membrane proteins, and their implications for the modulation of transport phenomena.

Experimental Approach:

Achievement of these objectives requires a multidisciplinary effort which we realize by contributing structural information to collaborative projects. At the level of specific techniques, we are engaged in:

1) high resolution electron microscopy.
2) image analysis and enhancement by optical diffraction and computer image processing.
3) development of appropriate specimen preparation techniques.

Statement of Progress:

The main thrust of our effort over the past year has been directed towards the design, appropriation and implementation of the requisite laboratory facilities.

a) Design and construction of a novel optical diffractometer. This instrument will enable both rapid, quantitative evaluation of electron micrographs in bulk and optical filtering of micrographs.

b) Renovation and equipment of electron microscope laboratory to accommodate a new high resolution Philips EM400 microscope. We have also modernized and streamlined the dark room facilities.

c) A comprehensive set of computer programs for image analysis, enhancement, and reconstruction of electron micrographs has been implemented on the IBM 370 computer of DCRT. These programs can be used interactively from a local terminal or, alternatively, run in batch mode. This amenity is being re-programmed to run on a PDP 11-70 minicomputer by Dr. B. Trus (N.D.I.), which adds the advantages of real-time use of image processing hardware installed in the Molecular Graphics unit of DCRT (television display, micro-densitometer, camera station). We are collaborating with Dr. Trus on this project.

Work has been initiated on the following specific projects.

I. Paracrystalline Tetragonal Particle Arrays in the Human Erythrocyte Membrane. (in collaboratioin with Dr. M. Gottlieb, LPB,NIAMDD)

We have observed patches of tetragonally disposed particles (periodicity approximately 14 nm) in erythrocyte membranes incubated in distilled water. Our immediate goals are chemical characterization of these particles, improvement of yield of these paracrystalline patches, and structural analysis of the several characteristically different appearances which the array projects in negatively stained preparations. In the longer term, we intend to extend the structural analysis to higher resolution by developing techniques to produce larger, better-ordered arrays, and to explore the possible functional implications of this aggregation phenomenon.

II. Structure Determination of Immunoglobin Crystals. (in collaboration with Dr. M. Navia, LMB, NIAMDD)

We have obtained preliminary results from processed images of negatively stained microcrystals of the IgG DoC, a human myeloma immunoglobin. This molecule's structure is known, to 6 Å resolution, from X-ray crystallography and we are currently evaluating the information content of the electron micrographs in terms of this model system. In the future, we intend to graduate to "combined" analyses of crystals of unknown immunoglobin molecules, using electron microscopy to locate the positions and orientations of the molecules within the unit cell and X-ray data for the high-resolution structure. We hope that this "hybrid technology" approach will facilitate structural determination of different immunoglobins and, by comparison, to afford some insight into the structural basis of antibody diversity.

III. The Ultrastructure of Beetroot Yellow Necrotic Virus.

Plant viruses represent some of the best known and experimentally most accessible systems for studies on nucleic acid-protein interactions. Characterizing the structure of the filamentous BYNV, our preliminary results indicate that we shall be able to determine the complement of protein, the coat protein/hair pair stoichiometry as well as the quaternary structure of the virus particle.

Significance to Biomedical Research and the Program of the Institute:

An improved understanding of macromolecular assembly events--in particular, membrane biogenesis and virus propagation--should be of direct relevance to several areas of medical research.

Proposed Course of Project:

Over the next year, we hope to complete the installation phase of the project and to actively pursue the specific experimental goals detailed above.

Publications:

Steven, A. C., Smith, P. R., and Horne, R. W.: Capsid fine structure of cowpea chlorotic mottle virus: From a computer analysis of negatively stained virus assays. J. Ultrastruct. Res. 64: 63-73, 1978.

Wurtz, M., Jacobsen, G. R., Steven, A. C., and Rosenbusch, J. P.: Paracrystalline arrays of protein-synthesis elongation factor Tu: Comparison with polymerized actin. Eur. J. Biochem. 88: 593-597, 1978.

Carrascosa, J. L. and Steven, A. C.: A procedure for evaluation of significant structural differences between related arrays of protein molecules. Micron 9: 199-206, 1978.

Steven, Alasdair C. and Carrascosa, Jose L.: Proteolytic cleavage and structural transformation: Their relationship in bacteriophage T4 capsid maturation. J. Supramol. Struct. 10: 1-11, 1979.

Annual Report of the Laboratory of Molecular Biology

National Institute of Arthritis, Metabolism and Digestive Diseases

The Laboratory of Molecular Biology has as its principal goal the understanding of biological processes at the molecular level. The research areas under investigation include the structures and chemical properties of biologically important materials. These involve studies of the organization of DNA and proteins in chromatin, investigations of enzyme and immunoglobulin structures by x-ray diffraction, investigation of polynucleotide chemistry and structure by synthetic and spectroscopic methods, studies of polynucleotide structure by fiber diffraction methods, and theoretical studies on the mechanism of energy conversion in biology, muscle contraction, the statistical thermodynamics of polynucleotide - complementary monomer interactions, ion transport and biochemical kinetics. In addition, other investigations are concerned directly with biological processes. These include the mechanism of transformation by SV40, the investigation of hemoglobin S gelation, genetic recombination, DNA synthesis and protein synthesis. Important advances have been made in these areas during this year.

Study of Functions Involved in Genetic Recombination

DNA Gyrase. We have continued our studies of DNA gyrase, the enzyme that is responsible for DNA supercoiling in prokaryotic cells. The specific sites of DNA breakage caused by the enzyme in the presence of oxolinic acid have been characterized. The two DNA strands are always broken four bases apart, with the 5'-ended single strand protruding and blocked by the protein. We have also cloned the genes coding for the subunits of DNA gyrase, which allows us to make the large quantities of enzyme needed for mechanistic studies.

Replication of Colicin E1 Plasmid DNA

Complete replication of colicin E1 plasmid (ColE1) DNA can be carried out in extracts of E. coli cells. The major products of this in vitro replication are completely replicated molecules and a unique type of early replicative intermediates containing a newly synthesized DNA fragment(s) in a small replication loop. The fragments have a sedimentation constant of approximately 6 S. Using L-strand specific 6S fragments which are synthesized first on ColE1 DNA, the origin of replication of ColE1 DNA has been determined. Replication initiates from one of three consecutive nucleotides in a unique region.

In vitro as well as in vivo replication of ColE1 DNA does not require ColE1 encoded proteins and depends entirely on bacterial functions. We fractionated active components for initiation of replication of the plasmid in cell extracts and found ribonuclease H is required in addition to DNA dependent RNA polymerase and DNA polymerase I. Results obtained with extensively purified enzymes show that these three enzymes are essential and sufficient for specific initiation of the plasmid DNA replication. The core enzyme of RNA polymerase and the large fragment of DNA polymerase I cannot replace respective holoenzymes. DNA synthesis requires twisted circular template

molecules. DNA synthesis by these enzymes terminates when these molecules relax. Addition of DNA gyrase allows further elongation of the strands. A transcript by RNA polymerase is probably processed by ribonuclease H and used as a primer for DNA synthesis which is carried out by DNA polymerase I. The superhelical turns are released by DNA replication. DNA gyrase is required for further elongation of the DNA strand which requires negative superhelical turns of the template molecules.

Plasmid RSF1030 is a plasmid whose replication depends on RNA polymerase and DNA polymerase I. We determined the nucleotide sequence around the origin of replication of the plasmid. The sequence around the RNA-DNA transition point of the plasmid is found to be identical to that of ColE1. Determination of nucleotide sequence around the origin of other related plasmids, p15 and ColDF13, is in progress.

Replication of the chromosome of E. coli bacteria can be initiated by ColE1 replication system when the plasmid integrates into the bacterial chromosome. This is shown by integrated suppression of replication of dnaAts bacteria by integration of ColE1 plasmid. Replication of the bacterial chromosome is now dependent on DNA polymerase I which is dispensable for normal replication of the chromosome.

Novel Recombination Systems

Tn9 is a transposable element, derived from a resistance transfer factor, which has the structure IS1-cat-IS1. We have shown that the directly repeated IS1 elements alone are responsible for Tn9 transposition. Recently, a mutant Tn9 has been isolated which transposes at 100-1000 times the normal frequency. The basis for this is being investigated.

Specialized Recombination in Bacteria

I have continued the study of the behavior of the insertion sequence γδ in Escherichia coli. The primary goal of these studies is an analysis of the genetics of the specialized recombination mediated by γδ. To that end, I have used an assay system in which the transposition of the γδ sequence to the R plasmid pBR322 is monitored by measuring the ability of the pBR322::γδ recombinant to be conjugally transferred by the plasmid F. Several mutants which appear to reduce the frequency of γδ transposition have been isolated. Characterization of these mutants, with particular regard to any effects they have on the transposition of other insertion elements and/or on other cellular functions, is in progress.

Studies are also being carried out to determine the mechanism of transposition of γδ. Preliminary results indicate the involvement of an F::pBR322 cointegrate plasmid as an intermediate in the transposition of γδ from F to pBR322. Models which predict that such a cointegrate structure is an intermediate in transposition have been proposed.

Finally, in collaboration with the Laboratory of Joan A. Steitz (Yale University) a DNA sequence analysis of γδ insertions has been carried out.

The results of these studies indicate that (1) $\gamma\delta$ is flanked by a 35 bp inverted repetition (which is related to the terminal sequences of other transposable elements; the implications of these sequence similarities is being investigated), (2) $\gamma\delta$ causes the duplication of a 5 bp target sequence at the site of insertion (a property similar to other insertion sequences), and (3) the presence of that 5 bp duplication is unnecessary for the ability of $\gamma\delta$ to transpose.

Genetics and Structure of the Oncogenic Virus, SV40

We continue to work on the molecular basis of transformation by Simian virus 40. SV40 encodes two proteins of 92K and 20K. We have demonstrated that the 20K protein is not required for tumor production since virus lacking this function when injected into newborn hamsters causes tumors. On the other hand, the tumors appear significantly later in life. The fact that the tumors were caused by the mutants has been verified by rescue of the virus and by immunoprecipitation of the 92K and 20K proteins. In vitro we have demonstrated that the 20K protein is required for the establishment of transformation in resting cells but not in growing cells. Cells transformed by the mutants are indistinguishable from cells transformed by the wild-type virus.

We have also isolated new mutants containing both a deletion that inactivates the 20K protein and a temperature-sensitive mutation of the 92K protein. Studies with the double mutants indicate that the initial induction of DNA synthesis by the virus occurs even at the nonpermissive temperature. Transformation studies suggest that only the 92K protein is required for the maintenance of transformation in certain media, but that the 20K function may be required for maintenance of transformation in other media.

Poly (I) Helix Formation. Dependence on Specific Complexing of Alkali Metal Ions.

We have found that helix formation by poly (I) is totally dependent on size-specific complexing to alkali metal ions or ammonium ion. The observed stability sequence is $K^+ > Rb$, NH_4^+, Cs^+, $> Na^+ >> Li^+$. The difference in Tm for the helix formed with K^+ and with Li^+ is $\sim 60°$. The binding sites are located in an axial hole or channel in the center of the four-stranded helix. Li^+ and Na^+ are bound in the plane of the four hydrogen-bonded inosine residues, complexed to each of four carbonyl oxygens at a distance of ~ 2.3Å. The larger ions are located on the axis midway between the planar tetramers ~ 2.8Å from each of eight oxygens. Both the Rb^+ and Cs^+ complexes form two distinct structures, characterized by CD and IR spectroscopy, both of which are four-stranded, with metals complexed in the axial channel. Rates of helix formation are very slow at low polymer concentration but can be greatly accelerated by freezing the solutions. The lower-melting forms (Form I) of the Rb^+ and Cs^+ complexes, however, are formed more rapidly ($t_{1/2} \sim 1$ hr) and are unaffected by freezing. Binding isotherms have been obtained for these latter complexes by ultraviolet spectroscopy.

Polyxanthylic Acid. Structures of the Ordered Forms

A number of studies of poly (X) have indicated that it pocesses an ordered form or forms but no specific structures for these have been proposed. The major difficulty has been that ionization at N3 in the neutral range places a negative charge upon each xanthime residue, leading to high electrostatic repulsion between the strands. Our studies indicate that the same phenomenon of internal metal ion complexing discovered for poly (I) can account for the existence of the poly (X) helix in neutral or basic solution. We conclude the poly (X) helix is four stranded, with the alkali metal ion complexed to the carbonyl groups lining the central cavity. Here the metal ions provide partial internal charge neutralization of electrostatic repulsion arising from ionization at N3. We have found marked specific alkali metal ion effects on the transition temperature of poly (X), though the magnitudes and pattern for the different metals are different from those observed with poly (I). In acid solution (pH < 5) poly (X) forms a different structure. Though the experimental evidence is not conclusive, it is consistent with the existence of a six-stranded structure at low pH.

Three-Dimensional Structures of Proteolytic Enzymes

The acid protease from Rhizopus chinensis has been refined, using the DCRT molecular graphics with the 'Bilder' program for model building, together with the Hendrickson-Konnert constrained least squares program for refinement. Since only partial sequence information is available, an attempt is being made to extract the sequence from the x-ray analysis. A tentative sequence has been assigned and modified on the basis of the x-ray data.

The application of the Hendrickson-Konnert constrained least squares program to the gamma-chymotrypsin data reduced the discrepancy factor between the 1.9 Å observed and calculated amplitudes to 20%.

Immunoglobulin Structure and Antibody-Antigen Interaction

The crystal structure of the Fab McPC 603 (a mouse IgA(k)) has been refined to 3.1 Å. Complete 2.7 Å data collected by the oscillation camera method are being processed by a program package developed in the laboratory. A re-analysis of the difference map between phosphorylcholine-soaked and native crystals confirms the absence of major conformational changes upon hapten binding to the Fab.

Data collection has been commenced for a high resolution analysis of the galactan binding J539 Fab. This will use the 4.5 Å model as a starting point, thus avoiding the necessity for further heavy atom data collection.

Chromatin Structure and Function

During the past year, we have continued to study the structure of chromatin, the complex of proteins and DNA found in eukaryotic nuclei. We have continued our detailed analysis of the arrangment of DNA in the chromatin subunit, the nucleosome.

Our earlier work had suggested that the DNA of the nucleosome might be quite accessible to the surrounding environment. We have confirmed this using the small chemical probe, dimethyl sulfate. This molecule reacts with the N7 of guanine in the large groove of DNA. We have measured the extent of this reaction at each nucleotide position within the 145 base pairs of the nucleosome core. Except for one exceptionally reactive region, there is no indication of any modulation of reactivity as a function of position within the nucleosome; the DNA of the nucleosome appears to be as accessible as DNA in solution. Similar conclusions can be drawn about reactivity in the small groove of DNA.

We have also been able to construct nucleosome-like particles using T4 phage DNA, in which the large groove is blocked by glucose residues. This again suggests that the large groove is blocked by glucose residues. This again suggests that the large groove is not involved in interactions with histones that are important in stabilizing nucleosomes. This and other evidence suggests to us that nucleosomes may be so designed as to leave the nucleotides of DNA accessible for biologically important reactions.

We have continued our studies of the role of the individual histone components in stabilizing the nucleosome. We had found earlier that histones H3 and H4 were necessary and sufficient to form a nucleosome-like structure. We have now shown that an octamer of H3/H4 is able to fold DNA of nucleosome size into a hydrodynamically compact structure.

As a tool for the study of the structure of transcriptionally active regions of chromatin, we have cloned a 6 kilobase genomic fragment containing the chicken adult β globin gene, and have mapped the fragment using restriction enzymes and electron microscopy. The gene has the same general pattern of inserted sequences seen in globin genes from other organisms analyzed in other laboratories.

We have begun a study of the possible role of DNA methylation in eukaryotic gene expression. Using restriction enzymes, a site near the 5' end of the globin gene has been shown to undergo remarkable changes in extent of methylation (5 position of cytosine) in cells which have a history of globin gene expression. In such cells, the site is completely unmethylated, while in all other cell types, the site is partly methylated. No systematic variation in methylation of other sites is seen. The possible role of this phenomenon in gene regulation is being investigated.

Statistical Thermodynamics of Polynucleotide - Complementary Monomer Interactions

A binding problem has been studied that has immediate application to muscle biochemistry and possibly future application to binding of ligands on polynucleotides. This is the problem of a linear array of binding sites which are competed for by a monovalent ligand and a divalent ligand. In the most general case, there are nearest-neighbor interactions, though in the muscle problem (myosin fragments bound on actin) interactions seem to be unimportant. Last year we studied the statistical distribution

problem, given the binding equilibrium constants. Currently, we are working out the statistical mechanical theory of the equilibrium constants themselves.

Energy Conversion in Biology, Especially Muscle Contraction

Four different topics have been studied under this project:

(a) An extensive study has been made of a four-state, single-site model of muscle contraction (recently submitted to Biophys. J.). This model represents both isometric transient and steady contraction data very well. Moreover, it conforms with current thinking on the biochemistry and kinetics of myosin-actin-ATP systems in solution.

(b) Our recent theoretical work on the influence of nearest-neighbor interactions on steady-state enzyme kinetics has been extended considerably. We have: considered small systems (enzyme lattices) with some empty sites and diffusion; studied the accuracy of the mean field approximation by making Monte Carlo calculations on the same systems; and investigated steady-state phase transitions that involve not only a conventional change in state but also a change in the dominant cycle of the kinetic diagram.

(c) A Monte Carlo and numerical integration study has been made of transient and steady-state kinetics of a complete model of the mitochondrial respiratory chain. This extends our related earlier work with Britton Chance. The model consists of four isopotential pools of electron-transfer components (usually four in each pool) and three four-state transducing site enzymes. More than 8000 distinct sub-states have to be taken into account in the theory and calculations. Also, in this field, we have studied the thermodynamic and kinetic implications of the recent four-system model for proton transport and oxidative phosphorylation, proposed by A. L. Lehninger. The four systems are: respiratory chain; reverse ATPase; ADP-ATP exchange; and H^+,P_i symport. We expect to pursue this topic further in the future, in collaboration with Dr. Lehninger.

(d) This work has dealt with generalizing the functions of classical thermodynamics to nonequilibrium steady states. The key function in the theory is a generalization of the entropy, called the sigma function. It is obtained from fluctuations in the extensive variables, and its Legendre transformations are maximized at steady state. These generalized thermodynamic functions have been used to characterize steady states in models of membrane transport, coupled enzymatically catalyzed reactions, and other nonlinear systems.

Work which examines the concentration fluxes in glycolysis is presently underway. the idea is to examine progressively more complicated segments of the glycolytic pathway in an effort to follow the progress of metabolism and regulation by allosteric enzymes.

Ion Transport Across the Nerve Axon Membrane

The work can be divided into two subjects. The first is concerned

with a general formalism for the calculation of small-signal admittance (or impedance) of membranes associated with the opening and closing kinetics of membrane channels. The differences in the admittance function between equilibrium and nonequilibrium channels are studied. Experimental data indicate that the potassium channels of squid axons may be in a nonequilibrium state.

The second topic is concerned with the study of "transport noise" associated with the translocation of individual ions within a channel. The movement of an ion may be described as a series of jumps over activation energy barriers and each jump contributes a single current pulse to the total external current. The investigation of transport noise could yield information on the rates of single ion transport steps within the channel.

Structural Features of mRNA and Their Influence on Translation.

The aim of this project is to determine the influence of specific structural features of the 5'-terminal portion of eukaryotic mRNA on initiation of protein synthesis in vitro. We have extended our studies using tobacco mosaic virus RNA and rabbit globin mRNA. These RNAs were enzymatically 'decapped' by nucleotide pyrophosphatase from potato, resulting in the removal of greater than 95% of 5'-terminal m^7G. Translation of control and 'decapped' mRNAs in a wheat germ or reticulocyte lysate showed dependence on 5'-terminal m^7G, especially at high potassium ion concentrations.

We have analysed the 5'-terminal cap structures of sub-genomic RNA fractions from turnip yellow mosaic virus (TYMV) RNA to determine whether there is 5' heterogeneity which would be indicative of minor species of mRNA coding for non-structural proteins. Enzymatic analysis of TYMV RNA suggests that in addition to m^7GpppG^mpA, and m^7GpppA^mpA, the expected structures for the genome RNA and coat protein mRNA, there is one other compound, whose structure is currently under investigation.

Protein Synthesis in Eukuryotic Systems: Mechanisms and Metabolic Controls

The long range objective of this project is to understand at the molecular level how proteins are synthesized in eukaryotic cells and how the process of protein synthesis is controlled at the levels of translation and transcription. As one approach toward our goals we have investigated aleurone layers of barley seeds incubated with gibberellic acid (GA). Aleurone cells have several features which make them ideal for the study of hormone action and gene activity. They are the only viable cells in deembryonated half seeds, they are nondividing, and they synthesize large amounts of a limited number of proteins in response to GA. We isolated RNA from aleurone cells and found that both total and poly $(A)^+$ RNA increase with time of incubation with GA, reaching a maximum around 12 hrs and thereafter declining. Total functional mRNA, as measured by quantitation of poly $(A)^+$ RNA with 5' caps, also shows a maximum at 12 hrs and parallels both the total in vitro translational capacity of poly $(A)^+$ RNA and its ability to direct the synthesis of α amylase in a wheat germ system. A possible mechanism of activation of mRNA by guanylylation and/or methylation at the 5' end was ruled out. Data with

inhibitors of RNA synthesis indicated that GA most likely stimulates protein synthesis by increasing the synthesis of mRNA. Studies with cycloheximide suggested that the induction of α amylase mRNA by GA requires the prior synthesis of some other protein(s).

Thermodynamics of Ligand Binding to Alcohol Dehydrogenase

The binding of NAD^+, NADH and ADP-ribose to horse liver alcohol dehydrogenase (LADH) has been studied calorimetrically as a function of pH at 25°. The enthalpy of NAD^+ binding varies with pH in a sigmoidal fashion and is -4.0 kcal $mol(NAD)^{-1}$ at pH 6.0 and +4.5 kcal mol $(NAD)^{-1}$ at pH 8.6 with an apparent pK_a of 7.6 $\pm$ 0.2. The enthalpy of proton ionization of the group on the enzyme is calculated to be in the range 8.8-9.8 kcal mol $(H^+)^{-1}$. In conjunction with the available thermodynamic data on the ionization of Zn-bound water in model compounds it is concluded that the group with a pK_a of 9.8 in the free enzyme and 7.6 in the enzyme-NAD^+ binary complex is, most likely, the zinc-bound water molecule. Our studies with the zinc-free enzyme provide further evidence for this conclusion.

Studies on the Solubility of Sickle Cell Hemoglobin

Our work has been dedicated to understanding the solubility behavior of sickle cell hemoglobin (HbS) from both an experimental and theoretical point of view since it has been reasoned that amelioration of the effects of sickling might be achieved by finding suitable physical conditions or a chemical reagent that would increase the solubility of HbS to an extent that would inhibit its polymerization within the time scale of the circulatory cycle of red blood cell deoxygenation and reoxygenation.

We have applied the scaled particle theory for mixtures of hard spheres to determine the effect of added proteins of varying size upon the solubility of sickle cell hemoglobin. Calculations based upon this model agree with experimental results for mixtures of normal and sickle cell hemoglobin (with A. P. Minton).

We have found that the relatively nontoxic chemical hexamethylenetetramine retarded the kinetics of polymerization and increased the solubility of HbS to a greater extent than any noncovalent inhibitor of gelation at comparable concentrations. These effects are due to the slight decomposition of HMT at neutral pH to form HCHO and NH_3. The increased solubility was attributed to the cross-linking of hemoglobin by HCHO and the increase in pH arising from the formation of NH_3. Additionally, we have shown that it is possible to cross-link hemoglobin within red cells and that this effect is enhanced in the presence of reducing agents such as ascorbic acid. Preliminary experiments to effect intracellular cross-linking of hemoglobin *in vivo*, in dogs, have not been successful.

Structural Studies on Polynucleotides

The aim of this project is to obtain structural information on polynucleotides and to provide insights into the factors which control structure, principally through *X-ray fiber* diffraction and model building

studies. Current areas include 1) determination of the helical parameters of DNA in concentrated solutions by diffraction techniques; 2) characterizing the secondary structures induced in DNA by a variety of solvents and conditions, using new sample-handling techniques which give ordered diffraction patterns even in the presence of large amounts of solvent.

Mammalian Enzymes Involved in Repair or Replication of DNA

The aim of this project is to identify and characterize enzymes involved in the repair and replication of DNA in higher organisms. Currently we are 1) Examining the ability of a eukaryotic DNA ligase to seal single strand breaks in chromatin; 2) Continuing to survey eukaryotic sources for a DNA gyrase-like activity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33000-13 LMB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Study of Functions involved in Genetic Recombination

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Martin Gellert,	Chief, Met. Enzymes Sec.	LMB NIAMDD
Other	Kiyoshi Mizuuchi	Visiting Associate	LMB NIAMDD
	Mark Guyer	Staff Fellow	LMB NIAMDD
	L. Mark Fisher	Guest Worker	LMB NIAMDD
	Mary O'Dea	Chemist	LMB NIAMDD

COOPERATING UNITS (if any)

Jun-ichi Tomizawa	Section on Molecular Genetics, LMB NIAMDD
Geoffrey Zubay	Dept. Biological Sciences, Columbia University, N.Y., NY

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Metabolic Enzymes

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4 1/3	3 1/3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

DNA Gyrase We have continued our studies of DNA gyrase, the enzyme that is responsible for DNA supercoiling in prokaryotic cells. The specific sites of DNA breakage caused by the enzyme in the presence of oxolinic acid have been characterized. The two DNA strands are always broken four bases apart, with the 5'-ended single strand protruding and blocked by the protein. We have also cloned the genes coding for the subunits of DNA gyrase, which allows to make the large quantities of enzyme needed for mechanistic studies.

PHS-6040
(Rev. 10-76)

Project Description:

DNA Gyrase We have continued our studies of E. coli DNA gyrase, an enzyme that induces supercoiling of double-stranded circular DNA in an ATP-dependent reaction. In the past year we have been investigating the properties of the homogeneous enzyme, an equimolar complex of two subunits determined by the cou and nalA genes respectively. The ATPase activity of the enzyme is strongly stimulated by relaxed (or nicked) circular DNA, but not by highly supercoiled DNA. Thus the ATP hydrolysis appears to be tightly coupled to the supercoiling reaction. The ATPase activity is totally inhibited by novobiocin, but not by oxolinic acid. Similarly, we have shown that an ATP-derived affinity label can be covalently attached to the smaller (Cou) subunit, and that this binding is blocked by novobiocin. Thus the Cou subunit is involved in the energy-coupling aspect of the supercoiling reaction. Previous work has shown that the DNA-relaxing activity, the other essential element of the overall reaction, is inhibited by oxolinic acid but not by novobiocin, and is thus primarily associated with the Nal subunit. The idea which we proposed some time ago, that the functions of the enzyme would be partitioned between its subunits, appears to be holding up.

We have also investigated further the aberrant double-strand cleavage of DNA produced by DNA gyrase in the presence of oxolinic acid. The cuts occur at specific sites, several of which have now been precisely located by DNA-sequencing methods. There is a uniform chemical specificity in that the two strands are always broken four bases apart, with the 5' ends protruding and blocked by protein. The DNA sequence specificity, however, has not yet been deciphered. Among the five cleavage sites that we have sequenced, there are no obvious homologies. Work on this problem is continuing.

Publications: Mizuuchi, K., Gellert, M., and Nash, H.A.: Involvement of supertwisted DNA in integrative recombination of bacteriophage lambda. J. Mol. Biol. 121: 375-392, 1978.

Mizuuchi, K., O'Dea, M. H., and Gellert, M.: DNA gyrase: subunit structure and ATPase activity of the purified enzyme. Proc. Natl. Acad. Sci. USA 75: 5960-5963, 1978.

Functions involved in specialized recombination in E. coli

The analysis of the specialized recombination in E. coli mediated by the transposable element γδ has been continued. Transposition of γδ from the plasmid F to the plasmid pBR322 is detected by examining the conjugal transmission of pBR322 determined drug resistance. Drug resistant transconjugants invariably are found to contain not pBR322 itself, but pBR322 containing an insertion of γδ. Much of our effort this past year has been directed to the isolation and characterization of host mutants in which γδ is incapable of transposing. At least two such mutants, detected as isolates which do not transfer drug resistance but which do transfer F, have been obtained. These

mutations are currently being mapped and compared with other mutations in E. coli which are incapable of carrying out specialized recombination involving both γδ and other transposable elements.

In collaboration with the laboratory of Joan Steitz at Yale University, γδ transposition has been analyzed by DNA sequencing techniques. These studies have shown that, upon insertion, γδ is associated with the duplication of a 5 base pair target sequence. The duplicated sequence is unrelated to the DNA sequence flanking γδ in the transpositional donor molecule, F. The sequence studies have also shown that γδ is bounded by a 35 base pair inverted repeat and that 27 of the nucleotides are identical to those bounding the transposable element Tn3. Hybridization studies have shown that there is homology between Tn3 and γδ at an internal region and functional relationships between γδ and Tn3 are being studied.

Finally, evidence has been obtained that an F::pBR322 cointegrate is an intermediate in the conjugal transfer of pBR322. Such a cointegrate has been proposed to be an intermediate in transposition of a translocatable element between two plasmids. As part of this project, we have found that γδ is present in the E. coli chromosome as well as on F. In one strain studied, two chromosomal copies were detected.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33001-10 LMB

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Protein Synthesis in Eukaryotes: Mechanisms and Metabolic Controls

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: Elizabeth S. Maxwell Research Chemist LMB NIAMDD

OTHER: Subbaratnam Muthukrishnan Visiting Scientist LMB NIAMDD

COOPERATING UNITS (if any)

Seed Laboratory, USDA, Beltsville, Md. (Dr. G. R. Chandra)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Metabolic Enzymes

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1.5	.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range of objective of this project is to understand at the molecular level how porteins are synthesized in mammals and how the process of protein synthesis is controlled at the levels of translation and transcription. The topics of current interest are: 1) the isolation and characterization of biologically active messenger RNA from eukaryotic cells. 2) The translation of eukaryotic mRNA in cell-free systems. 3) The identification and purification of immunoprecipitable forms of specific proteins synthesized in cell-free systems. 4) The conversion of possible precursors of secretory proteins to mature proteins. 5) The induction of mRNAs for specific proteins by plant hormones.

PHS-6040
(Rev. 10-76)

Project Description:

We have investigated gibberellic acid-induced changes in RNA isolated from aleurone layers of deembryonated barley seeds. This system has several features which make it attractive for the study of hormone action and gene activity. The aleurone cells are the only viable cells in deembryonated seeds. They are nondividing, and they synthesize large amounts of a limited number of proteins in response to the exogemeus addition of the plant hormone gibberellic acid. We examined the effects of gibberellic acid by following the quantitative changes in total RNA, functional mRNA, and the capacity of these RNAs to direct the synthesis of proteins in general, and α-amylase specifically, under standard in vitro conditions. We used the presence on RNA molecules of both 5' caps and 3' polyA ends as a criterion for functional mRNA. These two structures were detected by following the transfer of methyl groups from [^{3}H methyl] S-adenosylmethionine to the 2' OH of the penultimate nucleoside of the cap structure of RNA previously selected for poly(A) on oligodT cellulose. This specific methylation is mediated by a methyltranferase present in vaccinia virus. This procedure for determining functional message has not been described previously. It is useful for quantitating functional mRNA if the 2'OH of the penultimate nucleoside is normally unmethylated, as it is in wheat and barley.

Our results show that there is a continuing increase in total RNA as well as functional mRNA in aleurone cells during the first 12 hrs after gibberellic acid addition. This enhancement of functional mRNA is not the result of activation of an inactive pool of uncapped mRNA in the mature seeds. Inhibitors of RNA synthesis, such as cordycepin, reduce the amount of translatable mRNA that can be extracted, also suggesting that gibberellic acid-induced protein synthesis requires the continuing synthesis of mRNA. We observed a decline in total translational capacity of mRNA after 12 hrs of exposure to gibberellic acid, indicating that the induced mRNA is unstable, and that some control could also be exerted at the level of mRNA degredation. Somewhat surprisingly, the induction of the synthesis of α-amylase mRNA depends on protein synthesis, since it is blocked by cycloheximide. Thus, the action of gibberellic acid in the induction of mRNA for α-amylase may not be on the α-amylase gene per se, but on the synthesis of some other protein.

Publications: Iglewski, B.H., Sadoff, J., Bjorn, M.J. and Maxwell, E.S.: Pseudomonas aeruginosa exoenzyme S: An adenosine diphosphate ribosyltransferase distinct from toxin A. Proc. Natl. Acad. Sci. USA 75: 3211-3215, 1978.

Cox, G.S., Weintraub, B.D., Rosen, S.W., Krutzsch, C., Stotler, I. and Maxwell, E.S.: High molecular weight forms of human placental lactogen: Synthesis in vitro and binding to membrane receptors for lactogenic hormones. Arch. Biochem. & Biophys. 193: 355-367, 1979.

Chandra, G.R., Muthukrishnan, S. and Maxwell, E.S.: Hormonal regulation of genome activity of high plants. Presented at 13th Middle Atlantic Regional Meeting, March 1979. ACS monograph series, 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33005-03 LMB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structural features of mRNA and their influence on translation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Subbaratnam Muthukrishnan	Visiting Scientist	LMB NIAMDD
Other:	Elizabeth S. Maxwell	Research Biochemist	LMB NIAMDD
	Marjan Pleij	Guest Worker	LMB NIAMDD

COOPERATING UNITS (if any)

Institute of Biochemistry and Biophysics, Warsaw, Poland (Dr. Witold Filipowicz)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Metabolic Enzymes

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to determine the influence of specific structural features of the 5'-terminal portion of eukaryotic mRNA on initiation of protein synthesis in vitro. We have extended our studies using tobacco mosaic virus RNA and rabbit globin mRNA. These RNAs were enzymatically 'decapped' by nucleotide pyrophosphatase from potato which resulted in the removal of greater than 95% of 5'-terminal m^7G. Translation of control and 'decapped' mRNAs in a wheat germ or reticulocyte lysate showed dependence on 5'-terminal m^7G, especially at high potassium ion concentrations. We have analysed the 5'-terminal cap structures of sub-genomic RNA fractions from turnip yellow mosaic virus (TYMV) RNA to determine whether there is 5' heterogeneity which would be indicative of minor species of mRNA coding for non-structural proteins. Enzymatic analysis of TYMV RNA suggests that in addition to m^7GpppG^mpA, and m^7GpppA^mpA, the expected structures for the genome RNA and coat protein mRNA, there is one other compound, whose structure is currently under investigation.

PHS-6040
(Rev. 10-76)

Project Description

The main objective of this project is to identify the influence of specific structural features of eukaryotic mRNA on their biological activity.

The previous report on this project summarized our results on the effect of the m^7G residue and the 2' O-methyl and N^6methyl groups of cap structures of vaccinia mRNA on translation and binding of mRNA to ribosomes. We have extended our observations to other mRNAs namely tobacco mosaic virus (TMV) RNA and rabbit globin mRNA. Treatment with nucleotide pyrophosphatase, which removed > 95% of the m^7G portion of the cap, brings about a pronounced decrease of mRNA activity in both wheat germ extract and reticulocyte lysate at high K^+ ion concentrations. At low K^+ concentrations, the dependence is much less marked. The requirement for the 5'-terminal cap was found to be similar for the synthesis of the 130,000 and 165,000 molecular weight polypeptides coded by TMV RNA. This indicates that both proteins may be initiated at the common site close to the 5'-terminus.

We have looked at 5'-terminal heterogeneity in turnip yellow mosaic virus (TYMV) RNA by labelling the sub-genomic RNA using vaccinia 2'-O-methyltransferase, digesting the labelled RNA and analysing the 5'-terminal oligonucleotides. In addition to the expected products corresponding to the genome and coat protein mRNA, i.e. m^7GpppG^mpA and m^7GpppA^mpA, one other labelled compound was observed, suggesting the presence of a minor component RNA. The structure and source of this oligonucleotide are currently under investigation.

Publications: Keith, J. M., Muthukrishnan, S. and Moss, B.: Effect of methylation of the penultimate adenosine of capped mRNA on ribosome binding. J. Biol. Chem. 253: 5029-5041, 1978.

Wodnar-Filipowicz, A., Szczesna, E., Zan-Kowalczewska, M., Muthukrishnan, S., Szybiak, U., Legocki, A. B. and Filipowicz, W. 5'-Terminal 7-methylguanosine and mRNA function. Eur. J. Biochem. 92: 69-80, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33006-01 LMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

In vitro study of the site specific recombination of phage lambda.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: K. Mizuuchi Visiting Associate LMB NIAMDD

Other: M. Mizuuchi Guest Worker LMB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Metabolic Enzymes

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 2/3	2/3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have cloned attachment sites of phage λ on small plasmids. Such DNA molecules were used as the substrates for the intermolecular Int-promoted recombination reaction of phage λ in vitro. With this system we found that the phage attachment site (att P) and only att P, has to be on a supercoiled DNA molecule for an efficient reaction to take place. We generated shortened att P DNA and recloned such DNA fragments. By characterizing these att DNAs we found that about 200 base pair long sequence is involved in the specific recognition of att P. Further, those att fragments which have lost portions of att P sequence still can act as bacterial att site or pro phage att sites.

PHS-6040
(Rev. 10-76)

Project Description:

Integrative recombination reaction of phage λ is a reaction in which two specific DNA sites (att P and att B) are recombined to produce two hybrid sites (att L and att R). Previously we have shown that the supercoiled form of substrate is required for an efficient reaction.

We developed a series of new substrates for the study of this reaction by cloning fragments of DNA containing the att sites individually onto plasmid DNA molecules. These new substrates enabled us to study for the first time the intermolecular integrative recombination in vitro. By using this system we have shown that the supercoiling requirement is specific for the att P site. The att B site can be on a supercoiled, relaxed, or even linear DNA molecule.

To obtain further information about the structure of att DNA, we generated shortened att P DNA pieces by trimming a fragment containing the site and recloned these pieces as plasmids. We found that for the att P DNA to be functional, it has to retain its original sequence spanning about 200 base pairs. Those plasmid DNA molecules which have lost att P function can still retain some att functions. Such plasmid DNA's behave like att L, att R, or att B depending on which part of the att P sequence is missing.

Publications: Mizuuchi, L., Gellert, M. and Nash, H.A.: Involvement of supertwisted DNA in integrative recombination of bacteriophage λ. J. Mol. Biol. 121: 375-392, 1978.

Nash, H.A., Kikuchi, Y., Mizuuchi, K. and Gellert, M.: Integrative recombination of bacteriophage λ; genetics and biochemistry. In "Integration and Excision of DNA Molecules", ed. P. Hofschneider and P. Starlinger, Springer-Verlag Berlin Heidelberg, W. Germany, 1978, pp. 21-27.

Mizuuchi, K. and Mizuuchi, M.: Integrative Recomination of Bacteriophage λ; in vitro study of the intermolecular reaction. Cold Spring Harbor Symp. Quant. Biol. in press, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 33051-07 LMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mammalian Enzymes Involved in Repair or Replication of DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	S. B. Zimmerman	Research Chemist	LMB NIAMDD
Other:	B. H. Pheiffer	Chemist	LMB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.2	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to identify and characterize enzymes involved in the repair and replication of DNA in higher organisms. Currently we are 1) Examining the ability of a eukaryotic DNA ligase to seal single strand breaks in chromatin; 2) Continuing to survey eukaryotic sources for a DNA gyrase-like activity.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To isolate and characterize enzymes from mammalian sources which act on DNA, such as DNA kinase and DNA ligase, in order to attempt to understand more fully the processes of DNA repair and replication.

Methods: Standard techniques of biochemistry.

Major Findings: We wish to determine whether damaged sites in DNA tend to be localized with respect to nucleosomes in reconstituted chromatin, and also the influence such localization has on the enzymatic repair of these sites. The first DNA lesions being tested are single-strand breaks (nicks). The DNA is ^{32}P-labelled at chain termini; about 70% of this ^{32}P is at nicks and the remainder at ends and gaps. We are testing several enzymatic means of removing the ^{32}P from sites other than the nicks so that only the nicks will remain labeled. Limited phosphatase or S1 nuclease action may be satisfactory for this purpose; differential removal by a reversal of the DNA-kinase reaction was unsuccessful. Preliminary results with the labeled DNA indicate localization of the ^{32}P in reconstituted chromatin. A significant fraction of the ^{32}P at nicks is protected from micrococcal nuclease digestion and is isolated in monomer-like particles by sucrose-gradient centrifugation. The ^{32}P-labeled strands tend to be almost monomer length, indicating nicks tend to accumulate toward the ends of nucleosomal DNA. DNase I digestion of such samples gives a series of weak bands on single-strand gels. This is consistent with some preferential localization of nucleosomes relative to nicks, but we must demonstrate which fraction of the ^{32}P is involved in this behavior.

Ligation of the labeled nucleosomes with a eukaryotic enzyme proceeds to a definite limit that is lower than the limit of the enzyme on ^{32}P DNA extracted from these particles. This result as well as reconstruction experiments suggest that the ligase is sealing a limited number of sites within the nucleosome.

We have made further attempts, uniformly unsuccessful, to demonstrate a DNA gyrase activity in eukaryotic cell preparations.

Significance to Bio-medical Research and the Program of the Institute: The enzymes discussed above are thought to function in repair and replication of DNA. Damaged regions in DNA have been implicated in a variety of disease states as well as in aging and differentiation. Enumeration and characterization of the enzymes which carry out repair and replication will hopefully lend insight into the mechanisms of these processes and the aberrations in them which occur in various diseased states. Extension of these studies to repair in chromatin should more closely approach the *in vivo* state of DNA in higher organisms.

Proposed course: Attempts to increase the specifity of labeling of the nicked DNA will continue. If the proportion of ^{32}P in nicks can be increased from 70% to about 90%, we should be able to unambiguously decide if nicks are phasing nucleosomes under our conditions.

The localization of the sites of ligase action within nucleosomes may be determinable by exonuclease treatments of the sealed DNA reisolated from ligated nucleosomes. Such experiments with exonuclease III and λ exonuclease are in progress.

Reconstituted nucleosomes in which poly(dAdT)·poly(dAdT) replaces natural DNA have been described by Simpson's and by Olins' groups. These very uniform particles may allow a better resolution in the experiments currently being done with natural DNAs.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33053-07 LMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Thermodynamics of Hemoglobin S Gelation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.:	P. D. Ross	Research Chemist	LMB NIAMDD
Other:	S. Subramanian	Visiting Associate	LMB NIAMDD
	Allen P. Minton	Research Chemist	LBC NIAMDD
	Frank L. Brown, Jr.	Clinical Associate	CHB NHLBI
	James F. Harwell, Jr.	Veterinarian	LAMS NHLBI
	Robert M. Winslow	Sr. Investigator	CHB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) The scaled particle theory for mixtures of hard spheres has been applied to determine the desolubilizing effect of non-binding proteins of arbitrary size upon the solubility of Hb S. This methodology successfully accounts for the observed solubility behavior of Hb S in mixtures with other hemoglobin species. 2) We have found that the relatively nontoxic chemical hexamethylenetetramine retards the kinetics of polymerization and increases the solubility of HbS to a greater extent than any noncovalent inhibitor of gelation at comparable concentrations. These effects arise from the slight decomposition of HMT at neutral pH to form HCHO which cross-links the hemoglobin. We have shown that is possible to cross-link hemoglobin within red cells and that this effect is enhanced in the presence of reducing agents such as ascorbic acid. Preliminary experiments to effect intracellular cross-linking of hemoglobin in vivo, in dogs, have not been successful.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To gain a basic physical-chemical understanding of the sickling phenomenon and to interpret the behavior of potentially therapeutic additives in terms of their effects upon the kinetics and thermodynamics of gelation. Specifically: 1) quantitative evaluation of the extent of non-ideality of hemoglobin both in solution alone and in multicomponent systems. 2) A systematic survey of the effect of anions, cations and organic solutes upon the solubility of deoxyhemoglobin S so that an idea may be gained of the relative importance of the ionic, hydrophobic and hydrogen-bonding interactions stabilizing the microtubular structure of deoxyhemoglobin S and requirements for more effective inhibitors of gelation be determined. 3) In vitro and in vivo evaluation of potentially therapeutic solubilizers of HbS.

Significance: The scaled particle theory for mixtures of hard spheres provides a general methodology for dealing with non-ideal effects in the solution phase of mixtures of HbS with other proteins.

The work with hexamethylenetetramine provides a model for the examination of inhibitors of gelation of HbS. Studies have progressed from examination of pure hemoglobin solutions and determination of mechanism to red cells and finally testing in vivo.

Proposed Course of Project: Solubility studies aimed at finding more effective inhibitors will be continued. Attempts to verify our previous mechanism of non-covalent inhibition of gelation will be pursued by spectroscopic and x-ray crystallographic methods. Work directed towards effecting the slow release of hexamethylenetetramine in vivo will be carried out.

Publications: Ross, P.D., Briehl, R.N., and Minton, A.P.: Temperature dependence of nonideality in concentrated solutions of hemoglobin. Biopolymers 17: 2285-2288, 1978.

Minton, A.P. and Ross, P.D.: On the concentration dependence of the diffusion coefficient of hemoglobin. J. Phys. Chem. 82: 1934-1938, 1978.

Ross, P.D. and Subramanian, S.: Ionic and non-ionic effects on the solubility of deoxyhemoglobin S. In Caughey, W. (Ed.): Biochemical and Clinical Aspects of Hemoglobin Abnormalities. Academic Press, New York, 1978, pp. 629-646.

Ross, P.D. and Subramanian, S.: Hexamethylenetetramine: A powerful and novel inhibitor of gelation of deoxyhemoglobin S. Arch. Biochem. Biophys. 190: 736-748, 1978.

Ross, P.D. and Minton, A.P.: The effect of non-aggregating proteins upon the gelation of sickle cell hemoglobin: Model calculations and data analysis. Biochem. Biophys. Res. Comm., 88: 1308-1314, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33054-04 LMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structural Studies on Polynucleotides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: S. B. Zimmerman

Other: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this project is to obtain structural information on polynucleotides and to provide insights into the factors which control structure, principally through X-ray fiber diffraction and model building studies. Current areas include 1) determination of the helical parameters of DNA in concentrated solutions by diffraction techniques; 2) characterizing the secondary structures induced in DNA by a variety of solvents and conditions, using new sample-handling techniques which give ordered diffraction patterns even in the presence of large amounts of solvent.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Obtain structural information on polynucleotides and to provide insights into the factors which control structure.

Methods: Standard techniques of X-ray diffraction and biochemistry; new methods of sample preparation to examine oriented samples in the presence of excess solvent by X-ray diffraction; computerized molecular model building.

Major Findings; Several recent papers have suggested that DNA in solution does not exist in the exact 10-fold helix of Watson and Crick, but rather has about 10 1/2 base pairs per turn and has a quite different conformation from the Watson-Crick-Wilkins model for B DNA. As a test of these suggestions, we have measured the helical parameters of DNA in concentrated solutions by x-ray fiber diffraction methods. Fibers of the sodium salt of DNA were swollen with water within capillaries; the capillary served to limit water uptake and slowed dissolution. Samples containing up to 80% water gave essentially a B-form diffraction pattern and had virtually the same helical parameters [9.9 base pairs/turn (standard deviation = 0.14), 3.34 Å axial rise/residue (SD = 0.019)] as did the initial fibers [9.95 base pairs per turn (SD = 0.15), 3.33 Å axial rise per residue (SD = 0.015)]. Hence under highly solvated conditions where the interactions between molecules should be greatly reduced, DNA maintains its classical B-form structure.

DNA undergoes a reversible cooperative change in a number of its properties over a characteristic range of ethanol concentrations. This ethanol-induced transition of DNA has been studied by a number of groups, using circular dichroism as well as other assay techniques. There has been disagreement as to the species of DNA involved. We have used x-ray diffraction methods on DNA fibers exposed to an excess of solvent, as developed for the preceding study. The diffraction patterns serve as "fingerprints" to clearly show that the transition occurs between the B and A forms of DNA (at low and high ethanol concentrations, respectively) in agreement with the conclusions of some of the previous workers. At still higher ethanol concentrations, the diffraction pattern changes from that of the A form to that of a more disordered form.

Significance to Bio-Medical Research and the Program of the Institute: Polynucleotides are crucial in controlling cells in both normal and diseased states. Understanding of polynucleotide structure will hopefully be of aid in understanding and interacting with cells in such diseased states.

Proposed Course: There are many interactions which DNA and other polynucleotides undergo which can be profitably examined by the new sample-handling techniques which were developed above. For example, the C form of DNA has been stated to be present under certain solvent and salt conditions based upon indirect assay techniques. We can now directly test for its presence. Similar techniques may prove useful for studying drug - DNA interactions.

Publications: Steinert, P.M., Zimmerman, S.B., Starger, J.M. and Goldman, R.D.: Ten-nanometer filaments of hamster BHK-21 cells and epidermal keratin filaments have similar structures. Proc. Natl. Acad. Sci. USA 75: 6098-6101, 1978.

Zimmerman, S. B. and Pheiffer, B. H.: Helical parameters of DNA do not change when DNA fibers are wetted: X-ray diffraction study. Proc. Natl. Acad. Sci. USA 76: 2703-2707, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 33055-04 LMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Thermodynamics of Dehydrogenase-Ligand Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	S. Subramanian	Visiting Scientist	LMB NIAMDD
Other:	P. D. Ross	Research Chemist	LMB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The binding of NAD^+, NADH and ADP-ribose to horse liver alcohol dehydrogenase (LADH) has been studied calorimetrically as a function of pH at 25°. The enthalpy of NAD^+ binding varies with pH in a sigmoidal fashion and is -4.0 kcal $mol(NAD)^{-1}$ at pH 6.0 and +4.5 kcal $mol\ (NAD)^{-1}$ at pH 8.6 with an apparent pK_a of 7.6 ± 0.2. The enthalpy of proton ionization of the group on the enzyme is calculated to be in the range 8.8-9.8 kcal $mol\ (H^+)^{-1}$. In conjunction with the available thermodynamic data on the ionization of Zn-bound water in model compounds it is concluded that the group with a pK_a of 9.8 in the free enzyme and 7.6 in the enzyme-NAD^+ binary complex is, most likely, the zinc-bound water molecule. Our studies with the zinc-free enzyme provide further evidence for this conclusion.

Project Description:

Objectives: (1) To obtain thermodynamic parameters for the binding of NAD, NADH and ADP-ribose to liver alcohol dehydrogenase. (2) To examine the thermodynamic data in relation to the conformational changes in the enzyme and the nature of proton-dissociating group in liver alcohol dehydrogenase upon binding NAD. (3) To study the various enzyme-ligand interactions by ultra-violet difference spectroscopy using liver alcohol dehydrogenase with coenzymes and coenzyme analogs. (4) Examine the heat capacities of interactions of NAD, NADH, and ADP-ribose to several dehydrogenase at several temperatures.

Methods: Batch Microcalorimetry, Ultraviolet spectroscopy.

Major findings: (1) The binding of NAD^+, NADH and ADP-ribose to horse liver alcohol dehydrogenase (LADH) has been studied calorimetrically as a function of pH at 25°. The enthalpy of NADH binding is 0 ± 0.5 kcal mol^{-1} in the pH range 6-8.6. The enthalpy of NAD^+ binding, however, varies with pH in a sigmoidal fashion and is -4.0 kcal $mol(NAD)^{-1}$ at pH 6.0 and +4.5 kcal $mol(NAD)^{-1}$ at pH 8.6 with an apparent pK_a of 7.6 ± 0.2. The enthalpy of proton ionization of the group on the enzyme is calculated to be in the range 8.8-9.8 kcal $mol(H^+)^{-1}$ which indicates that the group with a pK_a of 9.8 in the free enzyme and 7.6 in the enzyme-NAD^+ binary complex is, most likely, the zinc-bound water molecule. Our studies with zinc-free enzyme provide further evidence for this conclusion. Therefore, the processes involving a conformational change of the enzyme upon NAD^+ binding and the mechanism of subsequent quenching of the fluorescence of Trp-314 must be reevaluated in the light of this thermodynamic study.

(2) The excess heat capacities of binding NAD, NADH and ADP-ribose to lactate dehydrogenases, alcohol dehydrogenases and malate dehydrogenase indicate that the ΔC_p values are moderately large negative quantities for all the enzymes studied indicating the formation of compact structures of the enzyme-ligand complexes. Liver alcohol dehydrogenase complexes, however, stand out from those of the other enzymes in that the thermodynamic parameters for the formation of complexes with the liver enzyme are, in general, more positive in enthalpies (over the temperature range of 5-35°C) than those for the other enzymes which are all characterized by very similar behavior as a group.

(3) The uv spectral studies of the complexes of liver alcohol dehydrogenase with NAD, NADH and ADP-ribose indicate that the coenzymes undergo an intramolecular destacking upon binding to the enzyme and that the coenzymes encounter a medium of low dielectric upon binding to the enzyme.

Significance to Biomedical Research and the Program of the Institute. This project is designed to obtain a better understanding of the relationship of structural similarities to function in a class of dehydrogenases by studying the thermodynamics of enzyme-ligand interactions using the method of microcalorimetry. The basic knowledge gained thereby could pave the way for unraveling some mechanistic aspects of glycolytic and metabolic enzymes.

Publications:

Ross, P.D. and Subramanian, S.: Hexamethylenetetramine: A powerful and novel inhibitor of gelation of deoxyhemoglobin S. Arch. Biochem. Biophys. 190: 736-743, 1978.

Subramanian, S. and Kaufmann, B. T.: Interaction of methotrexate, folates, and pyridine nucleotides with dihydrofolate reductase: Calorimetric and spectroscopic binding studies. Proc. Natl. Acad. Sci. USA 75: 3201-3205, 1978.

Weiter, J.J. and Subramanian, S.: Free radicals produced in human lenses by a biphotonic process. Investigative Ophthalmology and Visual Science 17: 869-873, 1978.

Subramanian, S., Stickel, D.C., Colen, A.H., and Fisher, H.F.: Thermodynamics of heterotropic interactions - the glutamate dehydrogenase NADPH glutamate complex. J. Biol. Chem. 253: 8369-8374, 1978.

Subramanian, S.: Thermodynamics of dehydrogenase reactions. Trends in Biochem. Sci. 4: 102-105, 1979.

Subramanian, S. and Ross, P.D.: The enthalpy of protolysis of liver alcohol dehydrogenase upon binding nicotinamide adenine dinucleotide J. Biol. Chem., 1979 in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 34001-14 LMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (30 characters or less)
Chromatin Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Felsenfeld	Chief, Sec. on Phys. Chem.	LMB NIAMDD
OTHER:	M. Behe	Guest Worker	LMB NIAMDD
	G. Ginder	Research Associate	LMB NIAMDD
	M. Goldsmith	Guest Worker	LMB NIAMDD
	J. McGhee	Staff Fellow	LMB NIAMDD
	J. Nickol	Staff Fellow	LMB NIAMDD
	G. Sandeen	Chemist	LMB NIAMDD
	L. Tack	Chemist	LMB NIAMDD
	W. Wood	Staff Fellow	LMB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Physical Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8	6.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

During the past year, we have continued to study the structure of chromatin, the complex of proteins and DNA found in eukaryotic nuclei. Our earlier work had suggested that the DNA of the nucleosome might be quite accessible to the surrounding environment. We have confirmed this using the small chemical probe, dimethyl sulfate. Except for one exceptionally reactive region, the DNA of the nucleosome appears to be as accessible as DNA in solution. We have also been able to construct nucleosome-like particles using T4 phage DNA, in which the large groove is blocked by glucose residues. As a tool for the study of the structure of transcriptionally active regions of chromatin, we have cloned a 6 kilobase genomic fragment containing the chicken adult β globin gene, and have mapped the fragment using restriction enzymes and electron microscopy. Using restriction enzymes, a site near the 5' end of the globin gene has been shown to undergo remarkable changes in extent of methylation (5 position of cytosine) in cells which have a history of globin gene expression.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The object of this project is to determine the structure of chromatin and the relationship of this structure to its biological role in cell differentiation.

Methods Employed: Spectrophotometry, analytical ultracentrifugation, dialysis equilibrium, and other standard techniques are used to study the binding of charged molecules to DNA, and the composition of the regions of DNA to which these molecules are bound. Standard chromatographic and electrophoretic techniques are used in purification and identification of histones. RNA polymerase is purified by classical enzymological techniques and its activity examined by membrane filtration, sucrose gradient sedimentation, or radioisotope incorporation. DNA-RNA hydridization methods are used to detect presence of specific polynucleotide sequences. Gel electrophoresis methods are used for measuring DNA and protein size, for examining nucleoprotein preparations, and for detecting chemically cross-linked proteins within chromatin. Recombinant DNA technology is employed to generate DNA fragments of defined sequence, or to insert eukaryotic DNA fragments into vectors capable of propagation in bacteria. All such work is carried out in appropriate and approved facilities under NIH guidelines. Xenopus oocytes are isolated and used for transcription studies.

Major Findings: Accessibility of DNA in nucleosomes - Our earlier work had suggested that the DNA of the nucleosome might be quite accessible to the surrounding environment. We have confirmed this using the small chemical probe, dimethyl sulfate. This molecule reacts with the N7 of guanine in the large groove of DNA. We have measured the extent of this reaction at each nucleotide position within the 145 base pairs of the nucleosome core. With the exception of one reactive region, there is no variation of reactivity within the nucleosome. Similar analyses of reaction with N3 of adenine lead to similar conclusions about accessibility of the small groove. The experiments have been repeated with the larger probe, diethyl sulfate, with identical results. We have studied the role of the DNA large groove in nucleosome formation, by making nucleosome-like particles using T4 phage DNA. This DNA has glucose residues blocking the large groove. Our preliminary data indicate that these do not interfere with nucleosome formation. All of this evidence suggests that the bases of DNA within nucleosomes are accessible to reaction with the environment, and that this property may be important to the functioning of nucleosomes.

Role of histones H3 and H4 - We have continued our studies of the role of individual histone components in stabilizing the nucleosome. We had found earlier that histones H3 and H4 were necessary and sufficient to form a nucleosome-like structure. We have now shown that an octamer of H3/H4 is able to fold DNA of nucleosome size into a hydrodynamically compact structure quite similar to a nucleosome containing the full histone complement.

Cloned chicken β globin gene - As a tool for the study of transcriptionally active chromatin, we have cloned a fragment of the chicken genome, 6.2 kilobase pairs long, containing the adult β globin gene. A detailed restriction enzyme map has been prepared, and the location of intervening sequences has been confirmed by R-loop mapping in the electron microscope. The positions and sizes of intervening sequences are similar to those found in globin genes from other organisms in other laboratories.

Methylation of DNA - We have studied the methylation of cytosine residues in the neighborhood of the adult β globin gene of chicken DNA. We find that DNA methylation is non-random, in that some sites can be completely methylated and others completely unmethylated. The pattern of methylation in the vicinity of the chicken beta-globin genes is tissue specific. In cells which are expressing or have expressed the adult beta-globin gene (adult marrow, reticulocytes and erythrocytes) one CCGG site close to the 5' end of the coding sequence and a second CCGG site close to the 3' end are completely unmethylated. In other tissues which are not expressing the adult β-globin gene (brain, oviduct, and red blood cells from 5-day old embryos) these sites are partially methylated. We believe these results to be the first correlation between site specific DNA methylation and eukaryotic gene expression.

Significance to Bio-medical Research and the Program of the Institute: Chromatin is the nucleoprotein complex that appears to contain the chemical information for cellular differentiation. Understanding of the organization of chromatin proteins on DNA and the way in which they affect transcription and other DNA functions in higher organisms will contribute to an understanding of how cells differentiate, and how they maintain this differentiated state, in which only a relatively small fraction of the total gene population of the cell is expressed. This is a central problem in cell biology.

Proposed Course of Project: We will study the organization of nucleoprotein in the neighborhood of the globin gene in avian red blood cells, using the cloned genomic β globin probe and subcloned fragments. We will also study the transcription of globin gene-containing plasmids using *Xenopus* oocytes. We will continue structural studies of the nucleosome core, its accessibility to various probes, and its stabilization under a variety of biologically interesting changes in the environment.

Publications:

Sollner-Webb, B., Melchior, Jr., W. and Felsenfeld, G.: DNase I, DNase II and Staphylococcal Nuclease Cut at Different, yet Symmetrically Located, Sites in the Nucleosome Core. *Cell* 14: 611-628, 1978.

Felsenfeld, G., Camerini-Otero, R.D., Simon, R., Sollner-Webb, B., Williamson, P. and Zasloff, M.: DNA Folding and Histone Organization in Chromatin. In Ahmad, R., Russell, T. R., Schultz, J. and Werner, R. (Eds.): Differentiation and Development, Miami Winter Symposia 15. New York, Academic Press, 1978, pp. 235-249.

Simon, R.H., Camerini-Otero, R.D. and Felsenfeld, G.: An Octamer of Histones H3 and H4 Forms a Compact Complex with DNA of Nucleosome Size. Nuc. Acids Res. 5: 4805-4818, 1978.

Williamson, P. and Felsenfeld, G.: Transcription of Histone-Covered T7 DNA by Escherichia coli RNA Polymerase. Biochemistry 17: 5695-5705, 1978.

Simon, R.H. and Felsenfeld, G.: A New Procedure for Purifying Histone Pairs H2A+H2B and H3+H4 from Chromatin Using Hydroxylapatite. Nuc. Acids Res. 6: 689-696, 1979.

McGhee, J. and Felsenfeld, G.: The Reaction of Nucleosome DNA with Dimethyl Sulfate. Proc. Natl. Acad. Sci. 76: 2133-2137, 1979.

Felsenfeld, G., McGhee, J., Sollner-Webb, B. and Williamson, P.: The Internal Organization of the Nucleosome. ICN-UCLA Symposium on Eucaryotic Gene Regulation, in press.

Ginder, G.D., Wood, W.I. and Felsenfeld, G.: Isolation and Characterization of Recombinant DNA Clones Containing the Chicken Adult β-Globin Gene. J. Biol. Chem., in press.

McGhee, J.D. and Ginder, G.D.: Specific DNA Methylation Sites in the Vicinity of the Chicken Beta-globin Genes. Nature, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 34002-15 LMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Proteolytic Enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. R. Davies	Chief, Sec. on Mol. Struc.	LMB NIAMDD
OTHER:	G. H. Cohen	Research Chemist	LMB NIAMDD
	E. W. Silverton	Research Chemist	LMB NIAMDD
	R. Bott	Staff Fellow	LMB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structure of the bovine proteolytic enzyme γ-chymotrypsin has been further refined at 1.9Å resolution by the method of restrained least squares as developed by Hendrickson and Konnert.

The acid protease from Rhizopus chinensis has been refined by a combination of restrained least squares and difference electron density maps to 2.0Å resolution. Adjustments have been made in the amino acid sequence by the protein based on the features apparent in the electron density map. A preliminary analysis has been made of the mode of binding of the universal acid protease inhibitor, pepstatin.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The determination of the three-dimensional structure of proteolytic enzymes, in particular, the structure of γ-chymotrypsin and of the acid protease from Rhizopus chinensis.

Methods Employed: X-ray diffraction analysis of single crystals including heavy atom isomorphous replacement, model building, coordinate refinement in both real and reciprocal space, and computer graphics.

Major Findings: 1. Our earlier refined model of γ-chymotrypsin has been subjected to the restrained least squares procedure of Drs. John Konnert and Wayne Hendrickson of the NRL. The resultant model is stereochemically superior to the previous model as well as being crystallographically more refined with R=20%. At this stage of refinement, features are sufficiently defined in fourier maps that we are able to make definitive statements about the molecular structure and the hydrogen bonding. The refined model is being compared with published coordinates for DIP-trypsin, benzamidine-trypsin, elastase and α-chymotrypsin. Considering backbone atoms of common residues only, the rms deviations of γ-chymotrypsin with α-chymotrypsin, elastase and the trypsins are 0.6, 0.8, 0.8 Å, respectively.

2. The previous tentative model of the acid protease from Rhizopus chinensis has been refined by a combination of restrained least squares refinement carried out in collaboration with Dr. Wayne Hendrickson of the NRL together with an examination of difference electron density maps utilizing the "Bilder" graphics program of Dr. R. Diamond. An initial tentative amino acid sequence was based on the overall appearance of the side chains in the multiple isomorphous replacement map, together with the sequence homology between the acid proteases that have already been sequenced (pepsin, chymosin and penicillopepsin). This sequence has been improved by the examination of new electron density maps with the refined phases. Although the refinement is not yet complete, it has been possible in many instances to replace the original amino acid assignment with one that gives a more satisfactory fit to the map.

The partially refined phases have also been used to produce an improved difference map showing the location of the universal hexapeptide acid-protease inhibitor, pepstatin. This inhibitor binds deep in the cleft between the two lobes of the protein, with the unusual amino acid, statine, located over the two active site aspartic acids, asp 32 and asp 215. The location of the subsite P_1 on the protein is fairly well defined as a large hydrophobic pocket. It is clear that, in order for the pepstatin to enter the cleft, there must be movement of the "flap" involving residues 75-80, since this flap overlaps the inhibitor.

Proposed Course: To refine the model of the acid protease to the highest resolution. To investigate the mechanism of action by inhibitor and low temperature studies.

Significance to Bio-medical Research and the Program of the Institute: Although the general mechanism of action of the serine proteases is fairly well understood, there is still some disagreement concerning the precise details. The refinement of γ-chymotrypsin at high resolution and different pH's should help resolve the problem.

The acid protease is one of a large class of enzymes that include pepsin, rennin, and the cathepsins. It has sequence homology with other members of the class and the elucidation of its structure should ultimately reveal the mechanism of action of these proteins.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 34003-11 LMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Crystal Structure Investigation of Antigen Antibody Interaction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. R. Davies	Chief, Sec. on Mol. Struc.	LMB NIAMDD
OTHER:	E. Padlan	Visiting Scientist	LMB NIAMDD
	M. Navia	Staff Fellow	LMB NIAMDD
	Y. Satow	Visiting Fellow	LMB NIAMDD
	G. Cohen	Research Chemist	LMB NIAMDD
	G. Gilliland	Staff Fellow	LMB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The model of McPC 603 Fab has been refined to 3.1Å resolution. Data have been collected for refinement to 2.7Å.

J539 Fab has been investigated by x-ray diffraction and by model building procedures.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: A comparative investigation of the crystal structure of the Fab fragment of immunoglobulin molecules that bind strongly to several different antigens. In this way, it is hoped to map out the binding site of antibodies and to illustrate the mechanism of antigen-antibody interaction.

Methods Employed: Preparation and analysis of pure materials. Crystallization and x-ray diffraction analysis with: a) heavy atom isomorphous replacement, b) rotation function, c) molecular replacement in real space. Model building. Computer comparison of structures. Computer automated refinement of phases and atomic coordinates.

Major Findings: The model of McPC 603 has been refined at 3.1Å resolution by the method of restrained least squares. Final maps with coefficients (2Fo-Fc) and (Fo-Fc) have been examined and changes have been made in the model to best fit these maps. A difference map of the phosphocholine bound to the Fab has been computed. In it, the largest peak is in the position where the choline would be expected to be located. This map demonstrates that no significant conformational change accompanies hapten binding.

The model of the combining site of the galactan-binding Fab J539 has been constructed in accordance with methods described previously. The coordinates of this model have been measured and will be compared with the model resulting from the current high resolution x-ray diffraction investigation. In this way it should be possible to assess the precision with which this kind of model can be constructed.

Proposed Course: 1. High resolution refinement of McPC 603 Fab will be completed. The phosphocholine binding will be analyzed further.

2. The high resolution (2.5Å) structure of J539 will be determined. The combining site will be compared with the model constructed from sequence data alone.

Significance to Bio-medical Research and the Program of the Institute: An understanding of the molecular basis of interaction between antibody and antigen is fundamental to our understanding of the immune response.

Publications:

Navia, M.A., Segal, D.M., Padlan, E.A., Davies, D., Rao, N., Rudikoff, S. and Potter, M.: Crystal structure of Mouse J539 galactan-binding immunoglobulin Fab at 4.5Å resolution. Proc. Natl. Acad. Sci. U.S.A., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 34004-02 LMB

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

DNA Supercoiling

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

CO-PI: Gary Felsenfeld, Chief Section on Physical Chemistry, LMB NIAMDD

Dan Camerini-Otero, Senior Surgeon GBB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Physical Chemistry

INSTITUTE AND LOCATION
NIAMDD, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

INACTIVE.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 35000-15 LMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Chemical and Structural Investigations of Nucleic Acids and Related Substances

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Todd Miles	Chief, Sec. on Org. Chem.	LMB	NIAMDD
OTHERS:	Frank B. Howard	Research Scientist	LMB	NIAMDD
	Wongil Limn	Visiting Fellow	LMB	NIAMDD
	Kunal B. Roy	Visiting Associate	LMB	NIAMDD
	Girjesh Govil	Visiting Associate	LMB	NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Organic Chemistry Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5 1/3	4 1/3	1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has the long range objective of studying the chemistry and structure of nucleotides, polynucleotides and nucleic acids and the application of these studies to the understanding of biochemical and biological processes. Topics of current interest include: (1) structure and reactivity of polynucleotides and the relation between the two; (2) stabilization of ordered forms of nucleic acids and polynucleotides; (3) spectroscopic studies of polynucleotides: infrared, ultraviolet, and circular dichroism; (4) design and synthesis of new polynucleotides having properties required for the foregoing studies.

PHS-6040
(Rev. 10-76)

Project Description:

Poly(I) Helix Formation Role of Size-Specific Complexing to Alkali Metal Ions

We reported last year the discovery of striking specific alkali metal ion effects on the stability of the ordered form of poly(I). We have pursued the subject this year and find that helix formation is completely dependent on size-specific metal ion (or NH_4^+) at a site other than the phosphate residues. The observed order of stability is $K^+ > Rb, NH_4^+, Cs^+ > Na^+ >> Li^+$. The specific binding sites are on the helix axis inside the axial cavity or channel formed by planar pairing of four inosine residues, with the carbonyl oxygens lining the cavity. The smaller cations (Li^+,Na^+) are in the plane of the bases with a coordination number (to the carbonyl oxygens) of four and a metal-oxygen and ligand distance of ~ 2.3Å for Na^+ and ~ 2.1Å and Li^+. The larger cations (K^+,NH_4^+,Rb^+,Cs^+) are bound midway between the planes of the bases with a coordination number (to the carbonyl oxygens) of eight and a metal-oxygen distance of ~ 2.8Å. Both Rb^+ and Cs^+ form two different helical complexes with poly(I) (Form I and Form II), distinguished by IR, CD, and transition temperature. With most of the ions under most conditions the rate of helix formation is very slow but is greatly accelerated by freezing the solution. With Form I of the Rb^+ and Cs^+ complexes, however, the rate is much faster and is unaffected by freezing. Spectrophotometric titration with Rb^+ or Cs^+ shows a sharp sigmoid dependence of absorbance on - log[M+].

Polyxanthylic Acid. Structures of the Ordered Forms

A number of studies of poly(X) have indicated that it possesses an ordered form or forms, but no specific structures for these have been proposed. The major difficulty has been that ionization at N3 in the neutral range places a negative charge upon each xanthine residue, leading to high electrostatic repulsion between the strands. Our studies indicate that the same phenomenon of internal metal ion complexing discovered for poly(I) can account for the existence of the poly(X) helix in neutral or basic solution. We conclude the poly(X) helix is four stranded, with the alkali metal ion complexed to the carbonyl groups lining the central cavity. Here the metal ions provide partial internal charge neutralization of electrostatic repulsion arising from ionization at N3. We have found marked specific alkali metal ion effects on the transition temperature of poly(X), though the magnitudes and pattern for the different metals are different from those observed with poly(I). In acid solution (pH<5) poly(X) forms a different structure. Though the experimental evidence is not conclusive, it is consistent with existence of a six-stranded structure at low pH.

8-Methyl Substituted Nucleotides and Polynucleotides

The nucleosides, 5'-monophosphates, and 5'-diphosphates have been synthesized for 8-methyl-2-aminoadenosine and 8-methyl guanosine. The diphosphate of the former has been polymerized with polynucleotide phosphorylase to make poly($2NH_2$8MeA). These molecules will be studied to determine the effect of steric interference of the 8-methyl group on syn-anti conformational equilibrium.

Publications:

Miles, H.T. and Frazier, J.: Infrared Spectroscopy of Polynucleotides in the Carbonyl Region in H_2O Solution. A·U Systems. Biochemistry 17: 2920-2927, 1978.

Pinnavaia, T.J., Marshall, C.L., Mettler, C.M., Fisk, C.L., Miles, H.T. and Becker, E.D.: Alakli Metal Ion Specificity in the Solution Ordering of a Nucleotide, 5'-Guanosine Monophosphate. Journal of the American Chemical Society 100: 3625-3627, 1978.

Miles, H.T., and Frazier, J.: Poly(I) Helix Formation. Dependence on Size-Specific Complexing to Alkali Metal Ions. Journal of the American Chemical Society 100: 8037-8038, 1978.

Mathelier, H.D., Howard, F.B., and Miles, H.T.: Circular Dichroism of Helices Formed by Purine Monomers with Pyrimidine Polynucleotides. Biopolymers 18: 709-722, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 35050-08 LMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Replication, Recombination and Repair of Microbial DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jun-ichi Tomizawa	Chief, Sec. on Mol. Genetics	LMB	NIAMDD
OTHER:	Tateo Itoh	Visiting Associate	LMB	NIAMDD
	Gerald Selzer	Staff Fellow	LMB	NIAMDD
	Tapan Som	Visiting Associate	LMB	NIAMDD
	Kazuo Yamaguchi	Visiting Associate	LMB	NIMADD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Genetics Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies on replication of plasmid colE1 DNA have been continued. Initiation of replication from a specific region of the plasmid can be carried out by purified enzymes. DNA dependent RNA polymerase, DNA polymerase I and ribonuclease H are essential and sufficient for the initiation. A transcript by RNA polymerase is probably processed by ribonuclease H and used as a primer for DNA synthesis which is carried out by DNA polymerase I. The superhelical turns are released by DNA replication. DNA gyrase is required for further elongation of the DNA strand to impose negative superhelical turns.

PHS-6040
(Rev. 10-76)

Project Description:

Complete replication of colicin E1 plasmid (ColE1) DNA can be carried out in extracts of E. coli cells. The major products of this in vitro replication are completely replicated molecules and a unique type of early replicative intermediates containing a newly synthesized DNA fragment(s) in a small replication loop. The fragments have a sedimentation constant of approximately 6 S. Using L-strand specific 6S fragments which are synthesized first on ColE1 DNA, the origin of replication of ColE1 DNA has been determined. Replication initiates from one of three consecutive nucleotides in a unique region.

In vitro as well as in vivo replication of ColE1 DNA does not require ColE1 encoded proteins and depends entirely on bacterial functions. We fractionated active components for initiation of replication of the plasmid in cell extracts and found ribonuclease H is required in addition to DNA dependent RNA polymerase and DNA polymerase I. Results obtained with extensively purified enzymes show that these three enzymes are essential and sufficient for specific initiation of the plasmid DNA replication. The core enzyme of RNA polymerase and the large fragment of DNA polymerase I cannot replace respective holoenzymes. DNA synthesis requires twisted circular template molecules. DNA synthesis by these enzymes terminates when these molecules relax. Addition of DNA gyrase allows further elongation of the strands. A transcript by RNA polymerase is probably processed by ribonuclease H and used as a primer for DNA synthesis which is carried out by DNA polymerase I. The superhelical turns are released by DNA replication. DNA gyrase is required for further elongation of the DNA strand which requires negative superhelical turns of the template molecules.

Plasmid RSF1030 is a plasmid whose replication depends on RNA polymerase and DNA polymerase I. We determined the nucleotide sequence around the origin of replication of the plasmid. The sequence around the RNA-DNA transition point of the plasmid is found to be identical to that of ColE1. Determination of nucleotide sequence around the origin of other related plasmids, p15 and ColDF13, is in progress.

Replication of the chromosome of E. coli bacteria can be initiated by ColE1 replication system when the plasmid integrates into the bacterial chromosome. This is shown by integrated suppression of replication of dnaAts bacteria by integration of ColE1 plasmid. Replication of the bacterial chromosome is now dependent on DNA polymerase I which is dispensable for normal replication of the chromosome.

Publications:

Itoh, T. and Tomizawa, J.: Initiation of replication of plasmid ColE1 DNA by RNA polymerase, ribonuclease H and DNA polymerase I. Cold Spring Harbor Symp. Quant. Biol. 43, 1979, in press.

Tomizawa, J. and Selzer, G.: Initiation of DNA Synthesis in Escherichia coli. Annual Review of Biochemistry, Vol. 48, 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 36002-05 LMB

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Novel Recombination Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Rosner	Research Biologist	NIAMDD:LMB
OTHER:	Mark Guyer	Staff Fellow	NIAMDD:LMB

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Microbial Genetics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/2	1-1/2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tn9 is a transposable element, derived from a resistance transfer factor, which has the structure IS1-cat-IS1. We have shown that the directly repeated IS1 elements alone are responsible for Tn9 transposition. Recently, a mutant Tn9 has been isolated which transposes at 100-1000 times the normal frequency. The basis for this is being investigated.

PHS-6040
(Rev. 10-76)

Objectives: An understanding in molecular terms of transposition, a process involving recombination between certain discrete DNA sequences (transposons) and non-homologous target chromosomes.

Major Findings: (1) We have demonstrated that transposition of Tn*9* (a 2600 base pair element in which IS*1*s flank in direct repeat the gene for chloramphenicol-resistance) is due solely to the IS*1* sequences.

(2) The presence of Tn*9* on bacteriophage λ (λ::Tn*9*) renders the λ itself transposable as IS*1*-λ-IS*1*. Thus, each end of each IS*1* on Tn*9* is functional.

(3) A λ::Tn*9* has been isolated which shows a 100-1000 fold higher frequency of transposition than the normal λ::Tn*9*.

(4) A simple system has been devised for screening chemicals for their ability to stimulate transposition of Tn*9*. Bacteria infected with λ::Tn*9* are plated on media containing chloramphenicol and a sample of the test compound is spotted on the plate. A semi-quantitative estimate of the extent of transposition can be made from the number of colonies which appear around the compound.

Significance to biomedical research: The discovery of transposable elements has led to a new understanding of how chromosomal rearrangements can occur. Transposition plays an important role in evolution, gene regulation and perhaps development. Transposition of drug resistance elements is of great importance in the epidemiology of infectious diseases and chemotherapy.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 36051-11 LMB

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Genetics and structure of the oncogenic virus, SV40

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert G. Martin, Chief, Section on Microbial Genetics, LMB NIAMDD

OTHER: Valerie Petit Setlow, Staff Fellow LMB, NIAMDD
Roland Seif, Visiting Fellow LMB, NIAMDD
Ana Chepelinsky, Visiting Associate LMB, NIAMDD

COOPERATING UNITS (if any)
Andrew Lewis, Research Scientist, LVD, NIAID
Charles Scher, Asst. Professor, Sidney Farber Cancer Institute

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Section on Microbial Genetics

INSTITUTE AND LOCATION
NIAMDD, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The early region of SV40 codes for two proteins of 20K and 90K. Deletion mutants that eliminate the 20K protein, temperature-sensitive mutants in the 90K protein and double mutants have been constructed. From studies with these mutants it is clear that the 20K protein is not required for viral replication. The onset of tumor appearance in hamsters inoculated with the deletion mutants is delayed, but tumors are found with high frequency. In tissue culture, the deletions affect the frequency of transformation in resting but not in growing cells. In appropriate growth factor deficient media a requirement for the 20K or for the 90K proteins for the maintenance of transformation can be demonstrated. Depending upon the cell lines used, a direct correlation between the condition of transformation and temperature-sensitivity of the resulting cell line are found following infection with mutants that are t.s. in the 90K protein. Analyses of the pattern of integrated SV40 in many cell lines, suggest that SV40 does not integrate as a circle, but perhaps only as a tandem linear molecule. The latter suggests that SV40 may replicate by the rolling circle mode in nonpermissive infections.

PHS-6040
(Rev. 10-76)

Project Description:

Objective: To analyze the molecular basis of transformation by SV40 using conditional lethal and viable deletion mutants.

Major Findings: (a) Inbred hamsters inoculated with the viable deletion mutants developed tumors, albeit at a later time than those inoculated with the wild-type virus. The tumor antigen patterns are characteristic of the deletions and virus rescued from the tumors were shown by restriction mapping to contain the deletions. The 20K tumor antigen is therefore clearly not essential for tumor formation. (Lewis and Martin)

(b) Double mutants induce host DNA synthesis at the nonpermissive temperature. Therefore the ts defect in the 90K protein is nonessential for this function (as is the 20K protein). This suggests either that there is a 3rd protein encoded by SV40, or that the primary defect in the 90K ts mutants is in their site-specific recognition of the origin of SV40 replicaton. (Setlow, DiLauro, Edwards, Seif and Martin).

(c) The 90K protein is known to be an autoregulatory protein. It is rapidly synthesized and degraded in tsA mutants incubated at the nonpermissive temperature. With the double mutants it has been possible to demonstrate that the 20K protein is nonessential for this autoregulation. (Setlow, DiLauro, Edwards and Martin).

(d) Transformation analyses have been carried out in partially defined media in order to assess the role of growth factors in transformation. Preliminary data suggest that the 20K protein partially complements for the absence of human platelet derived growth factor. (Scher and Martin).

(e) Transformation studies have been carried out in different cell lines with tsA mutants. In rat cells there appears to be a direct correlation between temperature-sensitivity of the transformants and active growth at the time of transformation. This correlation is less clear in mouse and hamster cells. (Seif and Martin)

(f) Analyses of a number of transformed cell lines by the blotting technique of Southern have been carried out. Of over 20 lines analyzed, none have less than a complete genome plus a partial tandem repeat of the integrated SV40 even though the cells were initially infected with multiplicities of virus less than 0.6. Single sites of integration have been found both in temperature-sensitive and temperature-insensitive lines. (Chepelinsky and Martin).

Publications

C. Chang, R.G. Martin, D. M. Livingston, S.W. Luborsky, C-P Hu, and P.T. Mora: Relationship between T-antigen and tumor-specific transplantation antigen in simian virus 40-transformed cells. J. Virol. 29, 69-75.

C.A.F. Edwards, G. Khoury and R.G. Martin: Phosphorylation of T-antigen and control of T-antigen expression in cells transformed by wild-type and tsA mutants of SV40. J. Virol. 29, 753-762, 1979.

A.M. Lewis, Jr. and R. G. Martin: The Oncogenicity of Simian Virus 40 deletion mutants that induce altered 17K t-proteins. Proc. Natl. Acad. Sci. USA. In press.

R. Seif and R. Martin: The growth state of the cell early after infection with SV40 determines whether the maintenance of transformation will be A-gene dependent or independent. J. Virol. (in press).

R.G. Martin, V.P. Setlow, and C.A.F. Edwards: The roles of the simian virus 40 tumor antigens in transformation of Chinese hamster lung cells; studies with SV40 double-mutants. J. Virol. In press.

R.G. Martin, V. P. Setlow, C.A.F. Edwards, and D. Vembu: The roles of the simian virus 40 tumor antigens in transformation of Chinese hamster lung cells. Cell. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 36101-05 LMB

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Energy conversion in biology, especially muscle contraction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Terrell L. Hill	Chief, Sec. on Theoretical Mol. Biol., LMB NIAMDD
OTHER:	Yi-der Chen	Research Chemist, LMB NIAMDD
	Teresa Chay	Guest Worker, LMB NIAMDD
	Joel Keizer	Visiting Scientist LMB NIAMDD

COOPERATING UNITS (if any)

Evan Eisenberg	Heart, LB
Leonard Stein	Heart, LB

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Theoretical Molecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Four different topics have been studied under this project:

(a) (Eisenberg, Hill, Chen) An extensive study has been made of a four-state, single-site model of muscle contraction (recently submitted to Biophys. J.). This model represents both isometric transient and steady contraction data very well. Moreover, it conforms with current thinking on the biochemistry and kinetics of myosin-actin-ATP systems in solution.

(b) (Hill, Stein, Chen) Our recent theoretical work on the influence of nearest-neighbor interactions on steady-state enzyme kinetics has been extended considerably. We have: considered small systems (enzyme lattices) with some empty sites and diffusion; studied the accuracy of the mean field approximation by making Monte Carlo calculations on the same systems; and investigated steady-state phase transitions that involve not only a conventional change in state but also a change in the dominant cycle of the kinetic diagram.

(c) (Hill, Chay) A Monte Carlo and numerical integration study has been made of transient and steady-state kinetics of a complete model of the mitochondrial respiratory chain. This extends our related earlier work with Britton Chance. The model consists of four isopotential pools of electron-transfer components (usually four in each pool) and three four-state transducing site enzymes. More than 8000 distinct sub-states have to be taken into account in the theory and calculations. Also, in this field, we have studied the thermodynamic and kinetic implications of the recent four-system model for proton transport and oxidative phosphorylation, proposed by A. L. Lehninger. The four systems are: respiratory chain; reverse ATPase; ADP-ATP exchange; and H^+,P_i symport. We expect to pursue this topic further in the future, in collaboration with Dr. Lehninger.

(d) (Keizer) This work has dealt with generalizing the functions of classical thermodynamics to nonequilibrium steady states. The key function in the theory is a generalization of the entropy, called the sigma function. It is obtained from fluctuations in the extensive variables, and its Legendre transformations are maximized at steady state. These generalized thermodynamic functions have been used to characterize steady states in models of membrane transport, coupled enzymatically catalyzed reactions, and other nonlinear systems.

Work which examines the concentration fluxes in glycolysis is presently underway. the idea is to examine progressively more complicated segments of the glycolytic pathway in an effort to follow the progress of metabolism and regulation by allosteric enzymes.

Publications:

Hill, Terrell L. and Eisenberg, Evan: Approximate theory of the Huxley-Simmons T_0,T_1, and T_2. In Cross-Bridge Mechanism of Muscle Contraction (ed) H. Sugi, University of Tokyo Press, 1979.

Hill, T. L. and Chen, Y.: Interacting enzyme systems at steady state: Further Monte Carlo calculations on two-state molecules. Proc. Natl. Acad. Sci. USA 75: 5260-5263, 1978.

Hill, T. L.: Effect of enzyme-enzyme interactions on steady-state enzyme kinetics. IV. Strictly steady-state examples. J. Theor. Biol. 75: 391-416, 1978.

Hill, T. L.: Steady-state phase or cooperative transitions between biochemical cycles. Proc. Natl. Acad. Sci. USA 76: 714-716, 1979.

Hill, T. L. and Stein, L.: Approximate steady-state properties of lattices of interacting three-state enzyme molecules: A novel phase transition. Proc. Natl. Acad. Sci. USA 76: 1014-1018, 1979.

Hill, T. L.: Coupled enzyme systems in a vesicular membrane: oxidative phosphorylation as an example. Proc. Natl. Acad. Sci. USA 76: 232-235, 1979.

Hill, T. L. and Chay, T.: Theoretical methods for the study of the kinetics of models of the mitochondrial respiratory chain. Proc. Natl. Acad. Sci. USA (in press).

Hill, T. L.: Steady-state coupling of four membrane systems in mitochondrial oxidative phosphorylation. Proc. Natl. Acad. Sci. USA (in press).

Keizer, Joel: Non-equilibrium thermodynamics and the stability of states far from equilibrium. Accounts Chem. Res. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AM 36102-08 LMB

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Statistical thermodynamics of polynucleotide - complementary monomer interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Terrell L. Hill Chief, Section on Theoretical Molecular Biology, NIAMDD

COOPERATING UNITS (if any)
Evan Eisenberg Heart, LB

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Theoretical Molecular Biology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/10	1/10	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A binding problem has been studied that has immediate application to muscle biochemistry and possibly future application to binding of ligands on polynucleotides. This is the problem of a linear array of binding sites which are competed for by a monovalent ligand and a divalent ligand. In the most general case, there are nearest-neighbor interactions, though in the muscle problem (myosin fragments bound on actin) interactions seem to be unimportant. Last year we studied the statistical distribution problem, given the binding equilibrium constants. Currently, we are working out the statistical mechanical theory of the equilibrium constants themselves.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 36103-08 LMB

PERIOD COVERED

October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Ion transport across the nerve axon membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Yi-der Chen Research Chemist LMB, NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Theoretical Molecular Biology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9/10	9/10	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES X☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The work can be divided into two subjects. The first is concerned with a general formalism for the calculation of small-signal admittance (or impedance) of membranes associated with the opening and closing kinetics of membrane channels. The differences in the admittance function between equilibrium and nonequilibrium channels are studied. Experimental data indicate that the potassium channels of squid axons may be in a nonequilibrium state.

The second topic is concerned with the study of "transport noise" associated with the translocation of individual ions within a channel. The movement of an ion may be described as a series of jumps over activation energy barriers and each jump contributes a single current pulse to the total external current. The investigation of transport noise could yield information on the rates of single ion transport steps within the channel.

PHS-6040
(Rev. 10-76)

ANNUAL REPORT OF THE LABORATORY OF BIOORGANIC CHEMISTRY

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

SECTION ON OXIDATION MECHANISMS

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

A systematic approach to the study of the cytotoxicity, mutagenicity, and carcinogenicity of the ubiquitous environmental carcinogen benzo[a]pyrene (BP) has been pursued consisting of i) synthesis of as many known and potential oxidative metabolites as was possible, ii) study of the metabolism of the hydrocarbon with these authentic standards in hand, iii) testing of these compounds for cytotoxic and mutagenic activity with bacterial and mammalian cells both in the presence and in the absence of added drug metabolizing systems such as cytochrome P-450 and epoxide hydrase, and iv) evaluation of the carcinogenicity of these synthetic standards in several animal models. These studies provided evidence which indicated that (+)-7β,8α-hydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene is the most important carcinogenic metabolite of benzo[a]pyrene. With the first identification of an ultimate carcinogenic form for any member of the polycyclic aromatic hydrocarbon class of carcinogens, the "bay-region" theory was formulated. This theory predicts that diol epoxides that have the epoxide group in the bay-region of the hydrocarbon will be the most chemically reactive and presumably most biologically active. To test the predictions of the theory, the hydrocarbons benzo[a]pyrene, benzo[a]anthracene, chrysene, dibenzo[a,h]anthracene, and 3-methylcholanthrene have been studied.

Benzo[a]pyrene (BP). In the previous report we described tumor studies which established that the metabolic pathway responsible for the major part of the carcinogenicity of BP consisted of i) initial formation of BP 7,8-oxide by the cytochrome P-450 system, ii) hydration to BP 7,8-dihydrodiol by epoxide hydrase, and iii) cytochrome P-450 catalyzed formation of 7,8-diol-9,10-epoxides. Initial tumor studies on mouse skin indicated that the (-)-7,8-dihydrodiol was more active than its (+)-enantiomer For this reason, the role of absolute stereochemistry in modulating tumorigenicity has been explored further with the newborn mouse tumor model. In this model, the (-)-diol is 16 to 60-fold more active than the (+)-enantiomer and induces lymphoma as well as lung adenoma. When the four metabolically possible enantiomeric diastereomers of the 7,8-diol-9,10-epoxides were examined in this model, only the (+)-7β,8α-diol-9α,10α-epoxide showed marked tumorigenicity. High activity for this isomer only had been predicted by mutagenicity tests with Chinese hamster V79 cells whereas the S. typhimurium tester strains had predicted high activity for all four isomers. The exceptional activity of the one isomer dramatically points out the importance of absolute stereochemistry, to carcinogenesis, a factor which was overlooked in the past. We have also synthesized (+)- and (-)-BP 7,8-oxide to test them in the newborn mouse model. BP 7,8-oxide is less active than BP in this tumor model as it was on mouse skin.

On oral administration, butylated hydroxyanisole was found to inhibit the carcinogenicity of both BP and BP 7,8-dihydrodiol. These results suggest that the inhibiting effect entails events which occur subsequent to the formation of the diol. The BP 7,8-diol-9,10-epoxides elicit damage to DNA which is recognizable by the ultraviolet excision repair system in normal human cells.

Benzo[a]anthracene (BA). Previous studies from our laboratories had shown that BA 3,4-dihydrodiol and 3,4-diol-1,2-epoxides were highly tumorigenic in accord with the predictions of the bay-region theory. However, other laboratories had claimed that the 3,4-dihydrodiol was not a metabolite of BA. Such has not proved to be the case. Careful chromatography of the liver microsomal metabolites of BA has established that the 3,4-dihydrodiol accounts for as much as 4% of the total metabolism. The fact that the amount of this metabolite is so small partially explains why BA is a somewhat weaker carcinogen than might have otherwise been expected.

Dibenzo[a,h]anthracene (DBA). The bay region theory predicts that the 3,4-dihydrodiol of DBA should be a proximate carcinogen of DBA. In initiation-promotion experiments on mouse skin (10 nmol), this dihydrodiol was found to be twice as active as DBA whereas the 1,2- and 5,6-dihydrodiols were inactive in causing the formation of papillomas. Results of tumorigenicity tests of these compounds in the newborn mouse model provided similar results. One particularly interesting aspect of these studies is the fact that the 5,6-dihydrodiol is a compound which acts as a false positive in the Ames Test since this compound can be activated to mutagens but is not carcinogenic.

3-Methylcholanthrene (MC). The 9,10-dihydrodiol of MC is predicted to be a proximate carcinogen of this hydrocarbon. Previously we reported that we were unable to detect this compound as a metabolite of MC. We were, however, able to isolate two diastereomeric 9,10-dihydrodiols of 1-hydroxy MC. One of these has proved to be from 5 to 100-fold more mutagenic to S. typhimurium strain TA 100 and to Chinese hamster V79 cells on metabolic activation than was MC. Since we pointed out the difficulty in isolating dihydrodiols from methylated hydrocarbons, other laboratories have sought and found hydroxymethylated dihydrodiols from 7-methyl BA and 7,12-dimethyl BA. These hydroxy-dihydrodiols are also thought to be proximate carcinogens.

Physical Organic and Synthetic Aspects of Drug Metabolism. In a continuing effort to relate chemical reactivity to biological activity, we have studied the hydrolysis of bay region diol epoxides of phenanthrene and chrysene in order to compare their reactivities to that of the corresponding BP derivatives. In all three cases, the diol epoxide-2 isomer with the benzylic hydroxyl group trans to the oxirane ring has proved more reactive in acid. In contrast, the diol epoxide-1 diastereomers are more reactive in the neutral to basic pH range possibly due to anchimeric assistance. Since tetrahydroepoxides are more reactive than their corresponding diol epoxides, conformation factors were suggested as major reasons for the differences in rate.

Benzo[e]pyrene is predicated to have more reactive diol epoxides than chrysene, yet is inactive as a carcinogen. For this reason we have synthe-

sized the two metabolically probably dihydrodiols of B[e]P in order to explore in detail this apparent exception to the bay-region theory.

Drug Metabolizing Enzymes. Studies have continued on the enzyme epoxide hydrase. Immunoprecipitated enzyme with antibody from four different sources retains full catalytic activity. Cross-reactivity studies have indicated that different species contain different forms of the enzyme. At present, it is still not possible to establish whether the highly purified preparation from rat liver constitutes a single polypeptide chain.

The studies have been conducted primarily by Drs. D. Jerina, H. Yagi, D. R. Thakker, R. N. Armstrong, M. Nordqvist, D. R. Buhler, M. Tada, B. Kedzierski, and Y. Ittah of this laboratory. A portion of the studies was done in collaboration with Drs. A. H. Conney, W. Levin, and A. W. Wood at Hoffman-LaRoche.

SECTION ON PHARMACODYNAMICS

Pharmacologically Active Compounds from Tropical Frogs and Other Natural Sources

Crystallization of an alkaloid ($C_{16}H_{29}NO$) from an Ecuadorean frog, Dendrobates tricolor, and X-ray diffraction analysis of a crystal has elucidated the last remaining major class of dendrobatid alkaloids. This compound, an indolizidine, was found to be 8-hydroxy-8-methyl-6-(2'-methylhexylidene)1-azabicyclo-[4.3.0]-nonane. Analysis of mass spectra and proton and carbon-13 magnetic resonance spectra allows the formulation of structures of six further members of this class of alkaloids including pumiliotoxin-A ($C_{19}H_{33}NO_2$) and pumiliotoxin-B ($C_{19}H_{33}NO_3$). An allo-series of alkaloids in this group contain a 7-hydroxy substituent. (Daly)

Alkaloids were analyzed in skin extracts from fourteen further species of frogs of the family Dendrobatidae. Frogs of the genus Phyllobates contained batrachotoxin while frogs of the genus Dendrobates contained at least four classes of simpler piperidine-based alkaloids. Frogs of the genus, Colostethus contained no toxins or, in the case of one Panamanian species, Colustethus inquinalis, a water soluble toxin of unknown structure. Significant amounts of pumiliotoxins and histrionicotoxins were found to be present in extracts of Phyllobates bicolor, a frog previously thought to contain only batrachotoxins. Phyllobates terribilis raised in captivity did not contain detectable amounts (< 50 ng) of batrachotoxin, while field-captured specimens contained initially 1 mg per frog and after 6 years in captivity 250 µg per frog. Apparently, dietary and/or environmental factors affect the production of batrachotoxin in this frog. The alteration in the sodium channel in nerve and muscle of Phyllobates, which confers protection to the action of batrachotoxin appears, however, to be genetic in origin since it still pertains in captive-raised frogs which do not contain batrachotoxin. All dendrobatid frogs, regardless of the type of toxin they produce or even whether or not they produce toxins, have similar structured granules in the secretory cells of the so-called cutaneous poison glands. It is tempting to

speculate that these cells serve or served a physiological function and that the synthesis, storage, and release of toxins is a secondary function which has evolved independently in many classes of amphibians.

Extracts from a previously undescribed species of dendrobatid frog, Dendrobates bombetes, contain three compounds whose mass spectra indicate that they represent a new class of dendrobatid alkaloids. The major compound has an empirical formula of $C_{16}H_{29}NO$ and yields a major mass spectral fragment of $C_6H_{13}N$. It is apparently a tricyclic compound with a tertiary nitrogen and a secondary hydroxyl group.

Pumiliotoxin-B, a representative of the pumiliotoxin-A class of dendrobatid alkaloids, has potent myotonic and cardiotonic activity. The molecular basis for such activity appears to derive from a facilitation of calcium-dependent processes; i.e., excitation-secretion coupling at presynaptic sites and excitation-contraction coupling at postsynaptic sites. In amphibian neuromuscular preparations this facilitation often results in calcium-dependent repetitive firing. Pumiliotoxin-B prolongs or facilitates calcium-dependent action potentials in molluscan and crustacean neurons. After prolonged exposure of amphibian preparations to pumiliotoxin-B, neuromuscular transmission, and to a lesser extent muscle action potentials and contracture, are impaired. These effects, unlike the myotonic and cardiotonic effects, are not readily reversible and appear to involve another site of action. (Nimitkitpaisan)

The binding of radioactive perhydrohistrionicotoxin to the acetylcholine receptor-ion channel complex in membranes from Torpedo electroplax is antagonized by histrionicotoxins > amantidine, local anesthetics > scopolamine, atropine. It would appear that effects of all of these compounds on ionic currents associated with the activation of acetylcholine receptors are due to interactions with the so-called histrionicotoxin-binding site on the channel.

Pumiliotoxin-C and synthetic analogs antagonize neuromuscular transmission apparently by blockade of nicotinic receptors. Acetylcholine-evoked end plate currents are blocked by these compounds, and irreversible binding of α-bungarotoxin to the receptor is antagonized. A synthetic analog with a propyl substituent at the 5-position on the cis-decahydroquinoline ring system is two-fold more potent than pumiliotoxin-C which contains a 5-methyl substituent. (Nimitkitpaisan)

The analgesic compound present in skin at 500 ng per frog in Dendrobates tricolor appears to have an empirical formula of $C_{10}H_{11}N_2OCl$ and, based on its ultraviolet spectrum, to contain a pyridine ring. The compound, while potent in vivo as an analgesic in the hotplate and Nielson tests. is relatively inactive in displacing radioactive dihydromorphine from brain membranes. The Straub-tail elicited in vivo by the compound is not effectively antagonized by naloxone.

Amphibians of the family Bufonidae contain high levels of compounds in skin which inhibit the binding of ouabain to an inhibitory site on Na^+-K^+-ATPase. In extracts from skin of toads of the genus Bufo and frogs of the

genus Atelopus, these compounds were identified as bufodienolides, while extracts of the frog Dendrophryniscus minutus contained more polar compounds which do not exhibit the ultraviolet chromophore characteristic of bufodienoldes. High levels of these compounds presumably serve in defense against predators. Skin extracts from many other species of frogs representing six other amphibian families contained low levels of compounds which inhibit binding of ouabain to Na^+-K^+-ATPase. Low levels of inhibitory compounds may represent physiological regulators for Na^+-K^+-ATPase in amphibian skin. All compounds were inhibitory to the enzyme. (Edwards)

Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

Specific antibodies to rat liver catechol-O-methyltransferase (COMT) were utilized to determine the localization of COMT at the cellular and subcellular level; to measure the rate of synthesis and degradation of the enzyme; and to measure and identify COMT in various hybrid cell lines. The initial study of the localization of COMT in non-neuronal glial elements and in ventricular ependymal cells of rodent brain is completed. Present results indicate that there are regular "breaks" in ependymal cell COMT suggesting that the postulated biochemical barrier for catechols is not continuous. A microtechnique for the direct assay of COMT activity was developed to confirm the immunohistochemical localization of COMT. The choroid plexus has been shown to contain 5 to 20 times more COMT activity than other areas of brain. (Creveling)

The exploration of the localization of COMT in the cardiovascular system has been expanded to include both arterial and venous elements from the aorta and vena cava to capillaries. The present studies indicate that COMT is present exclusively in endothelial cells and not in smooth muscle cells. Subcellular studies utilizing the peroxidase-antiperoxidase method at present appear to confirm this localization.

An initial study of the changes in the cellular localization of COMT in rat uterus during the course of pregnancy has been completed. Briefly, the enzyme is limited to epithelial cells of the uterus up to day 6. At day 7 with the implantation of the blastocyst cells containing COMT appear in the decidua in a characteristic geometric configuration on the antimesometrial side of the uterus. Two morphologically distinct decidual cells have been observed; one type was polygonal and the other stellate. Attempts to confirm this localization of COMT at the subcellular level utilizing the peroxidase-antiperoxidase technique has been inconclusive.

The extensive distribution of COMT in tissues responsive to steroid hormones has been of great interest, especially with regard to catechol steroids (2-hydroxyestradiol) and the elevated levels of COMT in human breast tissue. A marked elevation of COMT activity was observed in 20 malignant human breast tumors as compared to 9 non-malignant tumors. Immunohistochemical examination of these malignant tumors indicated that the COMT levels were increased primarily in the ductal tissue. The presence and localization of COMT by immunohistochemical techniques has been confirmed in breast tumors obtained from several rat and mouse strains with a high incidence of spontaneous

tumors. The localization again appears to be restricted to ductal tissue. Normal rat and mouse breast tissue contains very little COMT measured biochemically or histochemically. However, none of these animals was actively lactating mothers. In addition to demonstrating the presence of COMT in malignant tumors of breast tissue preliminary observations suggesting elevated levels of COMT have been made in human tumors from the colon, kidney, and Auerbach's plexus.

Rabbit anti-rat COMT has been used to measure COMT levels by immunotitration in a comparison of the levels of COMT during the development of two genetically different rat strains. One strain Fischer-344 rats have low COMT activity which is inherited in an autosomal recessive fashion. The Fischer-344 strain was compared with the Wistar-Furth strain. The levels of COMT in various organs were compared in newborn, weanlings, and adults. In general, the enzyme levels in kidney and liver were substantially higher than the levels in heart, brain, or blood in both strains throughout development. The increase of enzyme level with age was parallel in both strains. Comparisons of the immunotitratable COMT was parallel with COMT activity at all times. The temporal patterns of change in enzyme levels and activities were similar at multiple times during growth and development. Thus it is unlikely that temporal gene effects can explain the inherited differences in COMT activity in the two strains. It was suggested therefore that regulatory gene effects are responsible for the genetic differences.

Investigation of differences in the agonist specificities for the ring fluorinated derivatives of norepinephrine (NE) has been continued. It has been shown that 5-fluoronorepinephrine (5-FNE), 6-FNE, and NE are equipotent as alpha-adrenergic agonists in the isolated guinea pig aortic strip while 2-FNE has only marginal activity. In the isolated guinea pig atria, 2-FNE and NE are equipotent as beta-adrenergic agonists, 5-FNE is approximately 7 times more potent, while 6-FNE is essentially inactive. Examination of the effects of 2-, 5-, and 6-FNE on cardiac contractural force ($\beta 1$), arterial blood pressure ($\beta 2$) and femoral blood flow (α) were compared to those of NE in the anesthetized dog: 2-FNE acts on $\beta 1$ and $\beta 2$-adrenergic receptors, 5-FNE on α and $\beta 1$ receptors, and 6-FNE only on α-adrenergic receptors.

Studies of the potential anti-leukemic histidine analog, 2-fluorohistidine (2-FH) have continued. It is now evident the 2-FH mimics its parent amino acid, histidine, with regard to incorporation into protein in vitro (cell-free protein synthesis) or in vivo (mouse-L-cells). The behavior of 2-FH, blocking incorporation of histidine into protein but not affecting leucine incorporation in cell free systems, can best be explained by its replacement of histidine in conversion to the amino-acylated tRNA and subsequent incorporation into protein. The latter is in agreement with earlier studies on the incorporation of 2-FH into trichloroacetic acid-insoluble material in organ cultures of rat pineal glands and into murine proteins in vivo. Furthermore, polypeptide products synthesized in the Krebs II cell-free system in the presence of 2-FH appear to be identical in size and number to those synthesized in the presence of histidine.

Initial studies on the metabolic fate of [^{3}H]2-FH suggest the accumulation of a free metabolite in mouse tissue with an apparent half-life of 72 hours. Preliminary evidence indicates this metabolite to be 2-F-urocanic acid. In vitro studies have demonstrated the 2-F-urocanic acid is a powerful inhibitor of urocanase. A possible consequence of the formation of 2-F-urocanic acid in vivo may be a temporary interruption in the utilization of histidine as a one-carbon source for purine biosynthesis.

The Role of Benzodiazepine Receptors in the Nervous System

Inosine and hypoxanthine have been identified as endogenous compounds capable of altering benzodiazepine binding to receptors in vitro. Inosine and hypoxanthine compete for benzodiazepine binding sites at "brain specific" receptors but have no affinity for "kidney" (peripheral) receptors. Furthermore, both inosine and hypoxanthine cross-react with a diazepam (Valium)-specific antibody, suggesting a common recognition site by the immunologic system. Other purines, such as caffeine, also bind to the benzodiazepine receptor and these findings may represent a pharmacologic mechanism for the anxiogenic and convulsant properties of caffeine. The classic convulsant pentylenetetrazole was also found to interact with the benzodiazepine receptor at concentrations which are attained following a convulsant dose of this drug. (Skolnick)

Iontophoretic application of inosine to cultures of spinal cord obtained from fetal mice results in a dual action: The first is a rapid, depolarizing response which is also elicited by a water-soluble benzodiazepine, flurazepam. Inosine and flurazepam display cross-desensitization of this response, suggesting a similar agonist action at a receptor level. The second action of inosine is a slow hyperpolarization which is antagonized by flurazepam.

The importance of benzodiazepine receptors in mediating pentylenetetrazole induced convulsions was examined in mice. An excellent correlation was demonstrated between the number of "diazepam-occupied receptors" following a standard anticonvulsant dose of diazepam and the number of animals protected against convulsions by a standard convulsant dose of pentylenetetrazole. Furthermore, no more than thirty percent of receptors need be occupied to fully manifest anticonvulsant activity, suggesting the IC_{50} or Ki of a drug or endogenous ligand may not be a relevant measure of the ability of a compound to exert a pharmacologic or physiologic effect via the benzodiazepine receptor.

The role of the benzodiazepine receptor in mediating seizure activity was studied using two seizure models: pentylenetetrazole-induced convulsions and electroshock. A rapid increase in the number, but not affinity of benzodiazepine receptors was observed following tonic-clonic convulsions induced in both these models. This increase was fully manifest 15 minutes post-ictal and was no longer apparent 60 minutes post-ictal.

Binding of radioactive benzodiazepines to brain receptors was demonstrated in vivo. This was the first report of stereospecific, organ specific binding in vivo. The regional distribution of benzodiazepine binding in

vivo showed a perfect rank-order correlation with binding obtained in vitro. This technique may be of use in demonstrating drug/behavioral induced changes in the benzodiazepine receptor in vivo.

Intraventicular administration of inosine to mice afforded a partial protection from pentylenetetrazole-induced convulsions. This protection was expressed as an increase in the latency to convulsions following pentylenetetrazole rather than a reversal. Pharmacokinetic data supports the hypothesis that the transient action of inosine is due to a rapid metabolism and transport of this purine from the brain following intraventicular injection. Intraventricular administration of 2'-deoxyinosine, which is more potent than inosine in binding to benzodiazepine receptors resulted in a marked increase in latency of convulsions, while administration of 7-methylinosine and thymidine, both ineffective in displacing benzodiazepine binding in vitro, resulted in no alterations in latency. These findings support a benzodiazepine-like action of inosine, analogous to that which might be expected of an "ultrashort-acting benzodiazepine".

A simple and sensitive radioreceptor assay for benzodiazepines in plasma and other biological tissues was developed. The sensitivity obtained is equal to or greater than currently used technique of gas-chromatography with electron capture detection. In addition, the radioreceptor technique is rapid, requires no expensive equipment other than that available in most clinical laboratories, and gives a measure of the therapeutic equivalents of benzodiazepine which can be compared directly to diazepam. Patent rights for this technique are pending (issued to Drs. Skolnick and Paul through NIH).

Various models for studying the benzodiazepine receptor have been investigated, including mammalian retina, transformed cells of neural origin, and primary cultures of fetal brain and spinal cord. Data obtained from these models suggests that both the retina and primary cultures of brain and spinal cord may be used to study "brain specific" receptors in culture in order to examine the physiologic and pharmacologic function(s) of this receptor.

Irazepine, a noncompetitive, irreversible inhibitor of benzodiazepine binding is the first in a series of new synthetic compounds which will be utilized to investigate the structure and biochemical characteristics of the receptor.

The effects of alcohol dependence and withdrawal from alcohol on the benzodiazepine receptor have been examined. Small but significant reductions in benzodiazepine receptor number have been demonstrated in animals undergoing alcohol induced convulsions. (Williams)

Isolation of two substances from bovine brain and pituitary which inhibit diazepam binding to brain tissue in vitro has been accomplished. These compounds were not isolated from bovine liver or adrenal, suggesting a specificity to the central nervous system. These compounds are higher molecular weight than inosine or hypoxanthine; one component is only partially dialyzable, suggesting a molecular weight of 1000-5000 daltons.

Mixed brain gangliosides caused a 50-95% increase in the basal adenylate cyclase activity in membrane preparations from rat cerebral cortex. The activation was rapid and irreversible. The expression of activation showed a marked temperature dependence, with maximal effects seen in the 30-40° range and no activation seen at either 10° or 50°. The activation of adenylate cyclase by brain gangliosides was additive with respect to activation by fluoride and detergents, but was not additive with respect to activation by GppNHp or lysolecithin. The activation of the enzyme by gangliosides was unaffected by the presence of calcium ions, calcium-dependent activator protein, or EGTA. The addition of brain gangliosides to the membranes caused a consistent restoration of the responsiveness of the adenylate cyclase system to activation by β-adrenergic agents, an effect similar in magnitude to that observed by addition of guanyl nucleotides to the membrane preparations. (Partington)

Mixed brain gangliosides stimulated by about 1.5- to 2-fold the activity of rat cerebral cortical calcium-dependent and calcium-independent cyclic nucleotide phosphodiesterases in a concentration-dependent manner. The calcium-dependent enzyme was at least 10-times more sensitive to activation by gangliosides. Activation occurred rapidly and was readily reversible. Maximal activation of the calcium-dependent enzyme by gangliosides was only 50% of that elicited by lysolecithin or calcium-dependent activator protein. Activations by lysolecithin and gangliosides were apparently additive, while the activation by activator protein was slightly diminished in the presence of gangliosides. Trypsin-pretreatment of the calcium-dependent enzyme resulted in a loss of sensitivity to subsequent activation by gangliosides or activator protein without altering the activation by lysolecithin. Potassium ions inhibited basal activity of the calcium-dependent phosphodiesterase but had no effect on enzyme activated by gangliosides, activator protein, or lysolecithin. Phosphodiesterase in the membrane-bound state did not appear to be affected by gangliosides. It would appear that gangliosides may be involved in the regulation of both adenylate cyclase and phosphodiesterase in the central nervous system. (Davis)

Mild proteolysis of membrane preparations from rat cerebral cortex with low concentrations of endopeptidases such as trypsin or chymotrypsin caused a 50-400% increase in the basal adenylate cyclase activity. Maximal activation of adenylate cyclase was obtained by including the protease in the adenylate cyclase assay, although an activated preparation could be obtained by pretreatment of the membranes with chymotrypsin. The readily reversible activation of adenylate cyclase in membrane preparations by trypsin requires further investigation. The proteolytically activated enzyme retained responsiveness to activation by sodium fluoride and GppNHp, but was no longer activated by gangliosides or calcium dependent activator protein. The expression of activation of adenylate cyclase by protease treatment showed a marked temperature dependence, with maximal effects expressed in the 30-40° range and no activation expressed at either 10° or 50°. (Partington)

Incubation of rat cerebral cortical slices in Krebs-Ringer bicarbonate-glucose buffer induced a rapid decline in the responsiveness of the adenylate cyclase in subsequently prepared membrane preparations to stimulation by various activators of the enzyme. The desensitization of the adenylate cyclase system was time and temperature dependent, showed an absolute dependence on extracellular calcium ions, and was enhanced by the presence of serine proteases in the incubation medium, producing a membrane preparation which was partially responsive to activation by fluoride and GppNHp, but completely unresponsive to activation by ganglioside, proteolysis, or ß-adrenergic agonists. The nature of the calcium-dependent mechanism involved in the loss of responsiveness of membranal adenylate cyclase is unknown, but it appeared that the loss of sensitivity might be the result of the action of an extracellular, membrane-bound, calcium-dependent protease. It is remarkable that in the slices prior to homogenization no evidence for such a time and calcium-dependent loss of responsiveness of cyclic AMP-generating systems is manifest. (Partington, Edwards)

Adenosine interacts with two classes of sites which affect adenylate cyclase: Extracellular R-sites through which the enzyme can be stimulated or inhibited and intracellular P-sites though which the enzyme is inhibited. In rodent brain slices, adenosine 5'-cyclopropylcarboxamide (EC_{50} 1 µM) is a potent agonist at a stimulatory R-site. Competitive antagonists versus adenosine (100 µM) at the R-site include 5'-methylthioadenosine (IC_{50} 100 µM), theophylline (IC_{50} 60 µM), and 8-phenyltheophylline (IC_{50} 6 µM). Adenine arabinoside and 2',5'-dideoxyadenosine are relatively potent agonists at the P-site. Specific binding of the radioactive adenosine carboxamide or of radioactive adenine arabinoside represents 25-45% of total binding to crude synaptosomal membranes from various brain regions. However, displacement profiles by various agonists/antagonists are not consonant with pure R- or P-site ligand properties for either the carboxamide or the arabinoside. The high affinity binding site for the arabinoside (Kd 170 nM) is present in excess over a high affinity site for the carboxamide (Kd 10 nM) in rat cerebral cortical membranes. (Nimitkitpaisan, Bruns, Pons, Smellie)

The stereoisomers of N_6-phenylisopropyladenosine elicit accumulations of cyclic AMP in brain slices *via* interaction with adenosine-receptors. The response in guinea pig cerebral cortical slices and in rat hippocampal slices is blocked by theophylline, potentiated by biogenic amines and increased in the presence of a calcium chelator, EGTA. The *l*-isomer (EC_{50} 25 µM) is at least three-fold more potent than the *d*-isomer in eliciting accumulations of cyclic AMP in brain slices. In a rat coronal hippocampal slice *in vitro*, *l*-phenylisopropyladenosine reduces the amplitude of evoked synaptic responses generated *via* a monosynaptic pathway to the pyramidal neurons. The *d*-isomer is about 100-fold less potent. The difference in stereoselectivity with regard to activation of cyclic AMP-generating systems and with respect to inhibition of evoked synaptic potentials in hippocampal slices indicates that different adenosine-receptors are involved in these responses. (Smellie)

A series of 1,3-dialkylxanthines was examined as antagonists of adenosine-induced accumulation of cyclic AMP in guinea pig cerebral cortical slices and as inhibitors of brain phosphodiesterases. 8-Phenyltheophylline and 1,3-

dibutylxanthine appear to represent potent adenosine-antagonists with relatively low activity as phosphodiesterase inhibitors whereas 7-benzyl isobutylmethylxanthine is a potent and relatively selective inhibitor of the calcium-dependent phosphodiesterase with little activity as an adenosine-antagonist. None of the compounds was a potent inhibitor of the brain calcium-independent phosphodiesterase, although 1-isoamyl-3-isobutylxanthine might prove useful as an inhibitor of this enzyme because of its very low activity as an adenosine-antagonist. Indeed, isoamylisobutylxanthine markedly potentiates adenosine-elicited accumulations of cyclic AMP in brain slices. The potency of other phosphodiesterase inhibitors on the two major brain enzymes indicated that a 3,4-dialkoxybenzyl-2-imidazolidinone, RO 201724, and a 3,4-dialkoxyphenyl-2-pyrrolidone, ZK 62771, represent relatively selective inhibitors of the calcium-independent enzyme. (Davis, Smellie)

A calcium-dependent cyclic nucleotide phosphodiesterase from rat cerebrum was, in the absence of activator protein, inhibited by various monovalent cations. The inhibition was rapid, readily reversible, and concentration-dependent with 100 nM potassium ions inhibiting essentially all basal enzyme activity, while 100 mM sodium ions produced only moderate inhibition. At 100 mM, potassium and sodium inhibited enzyme activated by the calcium-dependent activator by only 15 to 30%, while at 55 mM no inhibition pertained. Potassium and sodium ions at 55 mM had no effect on the calcium-independent phosphodiesterase from rat cerebrum. The results indicate that at normal intracellular concentrations of potassium ions the activity of the calcium-dependent phosphodiesterase is completely dependent on the presence of calcium plus activator protein. (Davis)

A rapid, simple, and direct assay for 3',5'-cyclic nucleotide phosphodiesterase activity is based on the effective separation of cyclic AMP, cyclic GMP, or cyclic CMP from their corresponding 5'-nucleotides and nucleosides by chromatography on a polyacrylamide-boronate gel. The affinity of the boronate residue for cis-diols results in the retention of 5'-nucleotides and nucleosides while 3',5'-cyclic nucleotides are not retained. The coelution of all 5'-nucleotides and nucleosides allows for the accurate assessment of phosphodiesterase activity in preparations contaminated by other purine metabolizing enzymes such as 5'-nucleotidases and nucleotide and nucleoside deaminases. In most tissues nucleoside phosphorylase activity is very low and hence will not affect assays of phosphodiesterase activity based on use of these affinity gels. (Davis)

The accumulation of cyclic AMP elicited in guinea pig cerebral cortical slices by ATP or 5'-AMP is markedly antagonized by a combination of agents, $APCH_2P$ and GMP, which inhibit nucleotidase activity and by adenosine deaminase. Thus hydrolysis to adenosine appears requisite for the activation of cyclic AMP-systems in brain slices by adenine nucleotides. Only adenosine deaminase inhibits the accumulation of cyclic AMP elicited by depolarizing agents indicating that depolarizing agents cause primarily "release" of adenosine itself rather than ATP or other adenine nucleotide precursors of adenosine. (Pons, Bruns)

Glutamate at high concentrations elicits marked accumulations of cyclic AMP in guinea pig cerebral cortical slices. The glutamate-response is blocked completely by adenosine deaminase indicating a complete dependence on "release" of adenosine. The glutamate-response is not blocked by a specific glutamate antagonist, 1-hydroxy-3-aminopyrrolidone-2, indicating that it does not involve activation of the glutamate-receptors involved in the excitatory effects of glutamate on central neurons. Remarkably, the response to glutamate is potentiated by 2-chloroadenosine. It is proposed that glutamate causes both "release" of adenosine and other factors and that the latter potentiate the response of cyclic AMP-systems to "released" adenosine and to exogenous adenosine or 2-chloroadenosine. Such factors, like α-adrenergic agonists or H_1-histaminergic agonists are proposed to have no direct effect on cyclic AMP-systems. (Pons, Bruns)

Norepinephrine and 5-fluoronorepinephrine elicit a 15-fold accumulation of radioactive cyclic AMP in adenine-labeled rat cerebral cortical slices. The response to those agonists is partially blocked by either α- or β-antagonists, indicating a mixed α- and β-adrenergic activity for both compounds. Isoproterenol and 2-fluoronorepinephrine elicit an 8-fold accumulation of cyclic AMP which is antagonized by β-adrenergic blocking agents but not by α-adrenergic blocking agents. Responses to isoproterenol and 2-fluoronorepinephrine are not additive and appear to involve β_1-adrenergic receptors in rat cerebral cortex. 6-Fluoronorepinephrine elicits a 3-fold accumulation of cyclic AMP which is antagonized significantly by α-adrenergic blocking agents but not by β-adrenergic blocking agents. The response to 6-fluoronorepinephrine and the α-component of the norepinephrine-response shows certain of the characteristics of α_1-adrenergic receptors. Combinations of 6-fluoronorepinephrine with adenosine, with isoproterenol, or with 2-fluoronorepinephrine have greater than additive effects on cyclic AMP levels in rat cerebral cortical slices. The response to 6-fluoronorepinephrine is not significant if both adenosine- and β-receptors are blocked by the presence of 8-phenyltheophylline and propranolol. In guinea pig cerebral cortical slices, the norepinephrine derivatives have marginal effects on cyclic AMP levels. However, adenosine-elicited or histamine-elicited accumulations of cyclic AMP in guinea pig cerebral cortical slices are markedly potentiated by norepinephrine, 5-fluoronorepinephrine, and 6-fluoronorepinephrine but not by the β-adrenergic agonists, isoproterenol, or 2-fluoronorepinephrine. It is proposed that α-adrenergic agonists affect cyclic AMP-generating systems in brain slices solely by potentiation of the activation of such systems by adenosine, β-adrenergic, or histaminergic agonists. (Padgett, Creveling)

Binding of radioactive dihydroalprenolol to β_1-adrenergic receptors in rat cerebral cortical membranes is antagonized by norepinephrine, 5-fluoronorepinephrine and 2-fluoronorepinephrine. The 6-fluoro-analog of norepinephrine is at least 100-fold less potent. In contrast, binding of radioactive WB-4101 to α_1-adrenergic receptors in rat cerebral cortical membranes is antagonized by norepinephrine, 5-fluoronorepinephrine and 6-fluoronorepinephrine with 2-fluoronorepinephrine being 100-fold less potent. No such remarkable effects of the position of fluorine-substitution on the affinity of dopamines for β-adrenergic, α-adrenergic, or dopaminergic receptors were noted. Thus, it appears likely that interactions between the

benzylic hydroxyl and the 2-fluoro or 6-fluoro substituent are responsible for the lack of α-adrenergic or β-adrenergic activity, respectively in these compounds. (Nimitkitpaisan)

Norepinephrine, histamine, adenosine, glutamate, and depolarizing agents elicit accumulations of radioactive cyclic AMP from adenine-labeled nucleotides in particulate fractions from Krebs-Ringer homogenates of guinea pig cerebral cortex. The particulate fractions contain vesicular entities associated with a significant portion of the membranal adenylate cyclase. Particulate fractions from sucrose homogenates are a less effective source of such responsive entities. The responses to various agents are quite similar to responses which pertain with brain slices. Activation by norepinephrine is *via* α-adrenergic receptors, while activation by histamine is *via* H_1- and H_2-histaminergic receptors. Adenosine responses are potentiated by the amines and are antagonized by alkylxanthines. Glutamate and depolarizing agents elicit accumulations of cyclic AMP *via* "release" of endogenous adenosine. It is proposed, based on the absence of an α-adrenergic or H_1-histaminergic response in the presence of a combination of potent adenosine and H_2-histaminergic antagonists, that α-adrenergic and H_1-histaminergic receptor mechanisms do not activate adenylate cyclase directly in Krebs-Ringer particulate fractions, but merely facilitate activation by β-adrenergic, H_2-histaminergic, or adenosine-receptors. (McNeal, Creveling, Partington)

The accumulations of radioactive cyclic AMP elicited by adenosine, norepinephrine, and histamine in adenine-labeled vesicular entities of a particulate fraction from guinea pig cerebral cortex are greatly reduced as a result of prolonged preincubation. The presence of adenosine deaminase during preincubations largely prevents the loss of adenosine, norepinephrine, and histamine-responses. Adenosine deaminase was inactivated by deoxycoformycin prior to stimulation of cyclic AMP-accumulation by adenosine or amines. Adenosine deaminase cannot restore responsiveness of the cyclic AMP-generating systems. It is proposed that in particulate fractions of guinea pig cerebral cortex, low levels of adenosine cause a slow loss of both adenosine- and amine-receptors and/or of coupling of receptors to cyclic AMP-generating systems. (McNeal, Creveling)

Membrane potential of vesicular entities in Krebs-Ringer particulate preparations was assessed by measuring the rate of influx of radioactive methyltriphenyl phosphonium. Membrane depolarization by potassium ions caused the largest reduction in rate of influx, while selective neuronal depolarization by veratridine or batrachotoxin caused a lesser reduction. Ouabain also caused a reduction. Neither excitatory nor inhibitory neurotransmitters had any effect, nor did neurotransmitter-elicited accumulation of cyclic AMP affect the rate of influx of the phosphonium compound into the vesicular entities. (McNeal, Creveling)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 31100-14 LBC formerly Z01 AM 19400-13 LC

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TITLE OF PROJECT (80 characters or less)

Pharmacologically Active Compounds from Tropical Frogs and Other Natural Sources

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John W. Daly, Chief, Laboratory of Bioorganic Chemistry, NIAMDD

Other: Yuthasak Nimitkitpaisan, Staff Fellow, LBC, NIAMDD
Michael Edwards, Chemist, LBC, NIAMDD

COOPERATING UNITS (if any) C. W. Myers, Am. Museum of Nat. History, N.Y.C.; E. X. Albuquerque, M. E. and A. T. Eldefrawi, Univ. Md. School Medicine, Baltimore, Md.; I. L. Karle, U. S. Naval Res. Lab., Wash., D. C.; T. Tokuyama, Osaka City Univ., Osaka, Japan; E. Barrels de Bernal, Universidad de Valle, Cali, Colombia;

LAB/BRANCH
Laboratory of Bioorganic Chemistry

SECTION
Section on Pharmacodynamics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.5	0.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A total of over one hundred pharmacologically active alkaloids have been characterized in skin extracts of some 50 species of dendrobatid frogs. Five major classes have been eludicated and their pharmacology defined: 1) The batrachotoxins (6 compounds) are highly toxic, complex steroidal alkaloids which prevent the inactivation of voltage-dependent sodium channels in nerve and muscle; ii) The histrionicotoxins (10 compounds) are 3,7-disubstituted 1-azaspiro[5.5]undecanes, which facilitate the conversion of chemosensitive sodium-potassium channels in nerves and effector cells to the desensitized state; iii) The pumiliotoxin-C class (42 compounds) are 2,5-disubstituted decahydroquinolines, which block nicotinic receptors; iv) The pumiliotoxin-A class (24 compounds) are 6-alkylidene-8-hydroxy-8-methylindolizidines which facilitate and then block calcium-dependent excitation-secretion coupling and excitation-contraction coupling in nerve and muscle; v) The gephyrotoxins (11 compounds) are 3,5-disubstituted indolizidines and tricyclic analogs. Certain members of this class block voltage-dependent potassium channels and muscarinic receptors. An additional 14 compounds cannot be assigned to a particular class: one of these, a pyridine

Cooperating Units (Cont'd.): L. Tice, Laboratory of Experimental Pathology, NIAMDD; R. Highet, Laboratory Chemistry, NHLI: J. Flier, Beth Israel Hospital, Boston; L. Overman, Dept. Chemistry, Univ. California, Irvine; G. Brown, Neuroscience Program, University Alabama, Birmingham; W. Catterall, Univ. Washington, Seattle; B. Witkop, Laboratory of Chemistry, NIAMDD.

Summary of Work (Cont'd.): derivative, represents a new class of potent analgesics: Compounds antagonizing the binding of ouabain to Na^+-K^+-ATPase were identified as bufodienolides in extracts of certain bufonid toads and frogs, while a new class of Na^+-K^+-ATPase inhibitor was present in extracts of one bufonid frog.

Project Description:

Objectives: To elucidate the molecular basis for the pharmacological activity of batrachotoxin, histrionicotoxin, pumiliotoxins, and related substances. To isolate and elucidate the structures and molecular action of other pharmacodynamically active substances found in skin extracts of frogs and other natural sources. To explore biosynthetic pathways involved in the formation of these unique substances. To prepare radioactive ligands for the study of binding sites for these compounds.

Methods Employed: Sensitive bioassay methods, thin layer, column, high pressure liquid, and gas chromatography have been used in the purification of the active principles, while NMR, mass spectroscopy, X-ray crystallography, microchemical techniques, and radiochemical labeling are being utilized for structure elucidation and for the preparation of ligands for the study at binding sites. Isotopic tracer techniques have been used to study biosynthetic pathways. Biological activity has been investigated with both isolated enzyme systems, in intact cells, nerve, and muscle preparations and in intact animals.

Major Findings: Crystallization of an alkaloid ($C_{16}H_{29}NO$) from an Ecuadorean frog, Dendrobates tricolor, and X-ray diffraction analysis of a crystal has elucidated the last remaining major class of dendrobatid alkaloids. This compound, an indolizidine, was found to be 8-hydroxy-8-methyl-6-(2'-methylhexylidene)1-azabicyclo[4.3.0]-nonane. Analysis of mass spectra and proton and carbon-13 magnetic resonance spectra allows the formulation of structures of six further members of this class of alkaloids including pumiliotoxin-A ($C_{19}H_{33}NO_2$) and pumiliotoxin-B ($C_{19}H_{33}NO_3$). An allo-series of alkaloids in this group contain a 7-hydroxy substituent.

Alkaloids were analyzed in skin extracts from fourteen further species of frogs of the family Dendrobatidae. Frogs of the genus Phyllobates contained batrachotoxin while frogs of the genus Dendrobates contained at least four classes of simpler piperidine-based alkaloids. Frogs of the genus, Colostethus contained no toxins or, in the case of one Panamanian species, Colustethus inquinalis, a water soluble toxin of unknown structure. Significant amounts of pumiliotoxins and histrionicotoxins were found to be present

in extracts of Phyllobates bicolor, a frog previously thought to contain only batrachotoxins. Phyllobates terribilis raised in captivity did not contain detectable amounts (< 50 ng) of batrachotoxin, while field-captured specimens contained initially 1 mg per frog and after 6 years in captivity 250 μg per frog. Apparently, dietary and/or environmental factors affect the production of batrachotoxin in this frog. The alteration in the sodium channel in nerve and muscle of Phyllobates, which confers protection to the action of batrachotoxin appears, however, to be genetic in origin since it still pertains in captive-raised frogs which do not contain batrachotoxin. All dendrobatid frogs, regardless of the type of toxin they produce or even whether or not they produce toxins, have similar structured granules in the secretory cells of the so-called cutaneous poison glands. It is tempting to speculate that these cells serve or served a physiological function and that the synthesis, storage, and release of toxins is a secondary function which has evolved independently in many classes of amphibians.

Extracts from a previously undescribed species of dendrobatid frog, Dendrobates bombetes, contain three compounds whose mass spectra indicate that they represent a new class of dendrobatid alkaloids. The major compound has an empirical formula of $C_{16}H_{29}NO$ and yields a major mass spectral fragment of $C_6H_{13}N$. It is apparently a tricyclic compound with a tertiary nitrogen and a secondary hydroxyl group.

Pumiliotoxin-B, a representative of the pumiliotoxin-A class of dendrobatid alkaloids, has potent myotonic and cardiotonic activity. The molecular basis for such activity appears to derive from a facilitation of calcium-dependent processes; i.e., excitation-secretion coupling at presynaptic sites and excitation-contraction coupling at postsynaptic sites. In amphibian neuromuscular preparations this facilitation often results in calcium-dependent repetitive firing. Pumiliotoxin-B prolongs or facilitates calcium-dependent action potentials in molluscan and crustacean neurons. After prolonged exposure of amphibian preparations to pumiliotoxin-B, neuromuscular transmission, and to a lesser extent muscle action potentials and contracture, are impaired. These effects, unlike the myotonic and cardiotonic effects, are not readily reversible and appear to involve another site of action. (Nimitkitpaisan)

The binding of radioactive perhydrohistrionicotoxin to the acetylcholine receptor-ion channel complex in membranes from Torpedo electroplax is antagonized by histrionicotoxins > amantidine, local anesthetics > scopolamine, atropine. It would appear that effects of all of these compounds on ionic currents associated with the activation of acetylcholine receptors are due to interactions with the so-called histrionicotoxin-binding site on the channel.

Pumiliotoxin-C and synthetic analogs antagonize neuromuscular transmission apparently by blockade of nicotinic receptors. Acetylcholine-evoked end plate currents are blocked by these compounds, and irreversible binding of α-bungarotoxin to the receptor is antagonized. A synthetic analog with a propyl substituent at the 5-position on the cis-decahydroquinoline ring sys-

tem is two-fold more potent than pumiliotoxin-C which contains a 5-methyl substituent. (Nimitkitpaisan)

The analgesic compound present in skin at 500 ng per frog in Dendrobates tricolor appears to have an empirical formula of $C_{10}H_{11}N_2OCl$ and, based on its ultraviolet spectrum, to contain a pyridine ring. The compound, while potent in vivo as an analgesic in the hotplate and Nielson tests. is relatively inactive in displacing radioactive dihydromorphine from brain membranes. The Straub-tail elicited in vivo by the compound is not effectively antagonized by naloxone.

Amphibians of the family Bufonidae contain high levels of compounds in skin which inhibit the binding of ouabain to an inhibitory site on Na^+-K^+-ATPase. In extracts from skin of toads of the genus Bufo and frogs of the genus Atelopus, these compounds were identified as bufodienolides, while extracts of the frog Dendrophryniscus minutus contained more polar compounds which do not exhibit the ultraviolet chromophore characteristic of bufodienoldes. High levels of these compounds presumably serve in defense against predators. Skin extracts from many other species of frogs representing six other amphibian families contained low levels of compounds which inhibit binding of ouabain to Na^+-K^+-ATPase. Low levels of inhibitory compounds may represent physiological regulators for Na^+-K^+-ATPase in amphibian skin. All compounds were inhibitory to the enzyme. (Edwards)

Significance to Biomedical Research and the Program of the Institute: The exceptionally high toxicity of batrachotoxin, its selective action on nerve preparations, and its cardiotoxin properties gave the elucidation of its structure and correlations of structures with activity a high priority in biomedical research. Because of their remarkable effects on ion conductances, histrionicotoxin and various analogs provide unique tools for the study of the acetylcholine-modulated ion channels in electrogenic membrane. Gephryotoxin provides a new class of antagonists for potassium channels and muscarinic receptors. Antagonism of potassium channels by histrionicotoxins and gephryotoxins provides the basis for possible development of antiarrhythmic agents. The pumiliotoxin-A class of alkaloids provides an important new structure for the possible development of myotonic and cardiotonic drugs. Such compounds should prove invaluable tools for the investigation of calcium-mechanisms involved in transmitter release and muscle contracture. The pumiliotoxin-C class of alkaloids provides a new structure with curare-like activity. Other compounds from frog, fish, insect, and plant extracts also have physiological activities which warrant their structural and pharmacological investigation. Examples of such compounds occurring in frogs and toads include the samandarine, the bufodienolides, the catecholamines, indolealkylamines and histamines, and hypotensive and centrally active peptides. The potent analgesic effect of certain of the alkaloids is of particular relevance to the design of new drugs.

Proposed Course of Project: The structures of further alkaloids from frogs of the general Dendrobates, Colosthetus, and Phyllobates will be elucidated. The pharmacological activity of batrachotoxin, pumiliotoxin, gephyro-

toxin, and histrionicotoxin analogs will be studied in further detail. Radioactive batrachotoxin, gephyrotoxin, histrionicotoxin, and pumiliotoxin will be prepared and used to study their ultrastructural site of action. Further isolations of batrachotoxins, histrionicotoxins, pumiliotoxins, and related alkaloids will be carried out to provide material for chemical and pharmacological study. Pharmacologically active substances will be investigated in extracts from other families of tropical frogs, from fish, insects, and plants. Further synthetic analogs of dendrobatid alkaloids will be prepared. Extracts will be examined for the presence of new types of pharmacologically active peptides.

Publications:

Myers, C. W., Daly, J. W., and Malkin, B.: A dangerously toxic new frog (Phyllobates) used by Embera Indians of Western Colombia, with discussion of blowgun fabrication and dart poisoning. Bulletin Am. Mus. Nat. Hist., 161: 309-365, 1978.

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Myers, C. W., and Daly, J. W.: A name for the poison frog of Cordillera Azul, eastern Peru, with notes on its biology and skin toxins (Dendrobatidae). Am. Mus. Novitates 2674: 1-24, 1979.

Daly, J. W., Brown, G. B., Mensah-Dwumah, M., Myers, C. W.: Classification of skin alkaloids from neotropical poison-dart frogs (Dendrobatidae). Toxicon 16: 163-188, 1978.

Mensah-Dwumah, M. and Daly, J. W.: Pharmacological activity of alkaloids from poison-dart frogs (Dendrobatidae). Toxicon 16: 189-194, 1978.

APPENDIX

Contracts

Contract No. NO1-AM-4-2203: Isolation of Frog Toxins: $10,000; 1 man year.

Extracts of dendrobatid frogs provided by NIH have been fractionated to yield further quantities of batrachotoxins, histrionicotoxins, pumiliotoxins, and gephyrotoxins for pharmacological and chemical evaluation by NIH researchers. Structures of further congeneric alkaloids were determined by spectroscopic analysis. Derivatives of other alkaloids have been prepared for crystallization and spectroscopic analysis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 31101-11 LBC formerly Z01 AM 20000-10 LC

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. R. Creveling, Research Chemist, LBC, NIAMDD

Other: E. J. McNeal, Biologist, LBC, NIAMDD
W. Padgett, Biologist, LBC, NIAMDD

COOPERATING UNITS (if any) B. Hartman, Wash. Univ., St. Louis, MO.: C. Isersky, NIAMDD, ARB;R. Friedman, NIAMDD, LEP; R. T. Borchardt, Univ. Kansas, Lawrence; J. L. Costa, NIMH, LCS; L. Tice, NIAMDD, LEP; M. Lowe, NCI; K. Kirk, NIAMDD, LC; P. F. Torrence, NIAMDD, LC; L. A. Cohen, NIAMDD, LC; H. Seifried, Jitco, Rockville, MD.; E. Usdin,

LAB/BRANCH
Laboratory of Bioorganic Chemistry

SECTION
Section on Pharmacodynamics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the chemistry and biochemistry of biogenic amines, their amino acid precursors and transformation products. The areas of specific interest are: 1) the immunolocalization of catechol-O-methyl-transferase (COMT), monoamine oxidase (MAO) in malignant and normal human and rodent tissues. 2) The effect of substitution of fluorine on the aromatic ring of dopamine, norepinephrine, and serotonin of the biological properties of these amines. 3) The mechanism of toxicity and leukocytopoiesis of 2-fluorohistidine in normal and leukemic mice, and the metabolism and incorporation into protein of 2-fluorohistidine in vivo and in vitro.

PHS-6040
(Rev. 10-76)

Cooperating Units (Cont'd.): NIMH, PRB; R. M. Weinshilboum, Mayo Clinic, Rochester, MN.; K. Inoue, Chiba U., Chiba, Japan; X. Breakfield, Yale U., New Haven, CO.; J. D. Kohli, U. Chicago, IL; L. I. Goldberg, U. Chicago, IL.; A. M. Amin, NCI; U. Trendelenberg, U. Wurzburg, W. Germany; B. Bernard, G. D. Searle Co., Chicago, IL.: J. Ludford, NIDA, BB; R. Willett, NIDA, BB; G. Pryor, SRI, Menlow Park, CA.; E. McDaniel, NIAMDD, LNE; H. A. Krebs, Radcliffe Infirmary, Oxford, England; D. M. McCulloh, Univ. Maryland, College Park, MD.

Project description:

Objectives: To study the basic chemistry and biochemistry of the catecholamines, phenolic amines, indolealkylamines, imidazolealkylamines, and their precursors and metabolic transformation products. To develop new techniques for the investigation of membrane transport systems and receptor interaction as related to these amines and their interaction with various drugs. To study the mechanism of action of specific neurodegenerative agents structurally related to these amines; to investigate the specific sites of the cytotoxic action of these compounds and to describe both the subcellular morphological changes and specific interaction with macromolecules which accompany this cytotoxic action. To study the histopathology of the non-neuronal effects of these cytotoxic amine derivatives. To isolate and purify enzymes operative in the biosynthesis, metabolism, and physiological function of biogenic amines. To determine the physical properties of these enzymes. To prepare specific antibodies for these enzymes and related protein factors and apply the antibodies to the development of radioimmunoassays for the measurement and localization of these proteins. To investigate the distribution of these enzymes and proteins at both the cellular and subcellular levels through the application of immunoperoxidase, ferritin, and fluorescence techniques. To determine the effects of the introduction of fluorine and other substituents on the aromatic and imidazole rings of biogenic amines on transport, metabolic, and receptor-mediated systems for biogenic amines. To investigate the metabolism, toxic actions, and antineoplastic effects of various fluorine analogs of the amino acid precursors of certain biogenic amines and to ascertain the nature and mechanism of the incorporation of these analogs into protein in vivo and in various cultured cell lines. To devise and test a system for the quantitative measurement of rodent behavior and to correlate such measurements with function of biogenic amine systems in brain.

Methods Employed: Drugs, biogenic amines, and various precursors have been prepared with deuterium, iodine-125, carbon-14, and tritium to study the metabolism, transport, distribution, and release of these compounds. Standard methods of synthetic organic chemistry have been used to prepare these compounds. Standard methods of protein purification and analysis have been used to purify and isolate various enzymes and proteins, as well as specific affinity labeling techniques and immunoabsorption techniques. Standard methods of histopathology employing light and electronmicroscopy have been used to examine the cytotoxic effects of neurotoxic amines and certain cell specific toxic amino acid derivatives. Standard immunodiffusion, electrophoresis and immunization techniques have been employed in the development of various

antibodies. Standard methods of cell culture have been used for the testing of various amino acid analogs on various cell lines. New techniques for the fixation of tissues to facilitate the localization of antibody-specific proteins have been developed. A new system for the quantitative analysis of rodent behavior has been explored and has required the development, through a trial and error procedure of computer analysis to interpret the validity of the behavioral parameters.

Major Findings: Specific antibodies to rat liver catechol-O-methyltransferase (COMT) are continuing to be utilized to determine the localization of COMT at the cellular and subcellular level; to measure the rate of synthesis and degradation of the enzyme; and to measure and identify COMT in various hybrid cell lines. The initial study of the localization of COMT in non-neuronal glial elements and in ventricular ependymal cells of rodent brain is completed. Confirmation of the immunofluorescent localization utilizing the immunoperoxidase histochemical technique has yielded identical results. Further this technique is being utilized to determine the subcellular localization of COMT in cells of the choroid plexus of rat. Present results indicate that there are regular "breaks" in ependymal cell COMT suggesting that the postulated biochemical barrier for catechols is not continuous. A microtechnique for the direct assay of COMT activity was developed to confirm the immunohistochemical localization of COMT. The choroid plexus has been shown to contain 5 to 20 times more COMT activity than other areas of brain.

The exploration of the localization of COMT in the cardiovascular system has been expanded to include both arterial and venous elements from the aorta and vena cava to capillaries. The present studies indicate that COMT is present exclusively in endothelial cells and not in smooth muscle cells. Subcellular studies utilizing the peroxidase-antiperoxidase (PAP) method at present appear to confirm this localization.

An initial study of the changes in the cellular localization of COMT in rat uterus during the course of pregnancy has been completed. Briefly, the enzyme is limited to epithelial cells of the uterus up to day 6. At day 7 with the implantation of the blastocyst cells containing COMT appear in the decidua in a characteristic geometric configuration on the antimesometrial side of the uterus. Two morphologically distinct decidual cells have been observed; one type was polygonal and the other stellate. Attempts to confirm this localization of COMT at the subcellular level utilizing the peroxidase-antiperoxidase technique has been inconclusive.

The extensive distribution of COMT in tissues responsive to steroid hormones has been of great interest, especially with regard to catechol steroids (2-hydroxyestradiol) and the elevated levels of COMT in human breast tissue. A marked elevation of COMT activity was observed in 20 malignant human breast tumors as compared to 9 non-malignant tumors. Immunohistochemical examination of these malignant tumors indicated that the COMT levels were increased primarily in the ductal tissue. The presence and localization of COMT by immunohistochemical techniques has been confirmed in breast tumors obtained from several rat and mouse strains with a high incidence of spontaneous

tumors. The localization again appears to be restricted to ductal tissue. Normal rat and mouse breast tissue contains very little COMT measured biochemically or histochemically. However, none of these animals was actively lactating mothers. In addition to demonstrating the presence of COMT in malignant tumors of breast tissue preliminary observations suggesting elevated levels of COMT have been made in human tumors from the colon, kidney, and Auerbach's plexus.

Rabbit anti-rat COMT has been used to measure COMT levels by immunotitration in a comparison of the levels of COMT during the development of two genetically different rat strains. One strain Fischer-344 rats have low COMT activity which is inherited in an autosomal recessive fashion. The Fischer-344 strain was compared with the Wistar-Furth strain. The levels of COMT in various organs were compared in newborn, weanlings, and adults. In general, the enzyme levels in kidney and liver were substantially higher than the levels in heart, brain, or blood in both strains throughout development. The increase of enzyme level with age was parallel in both strains. Comparisons of the immunotitratable COMT was parallel with COMT activity at all times. The temporal patterns of change in enzyme levels and activities were similar at multiple times during growth and development. Thus it is unlikely that temporal gene effects can explain the inherited differences in COMT activity in the two strains. It was suggested therefore that regulatory gene effects are responsible for the genetic differences.

Investigation of differences in the agonist specificities for the ring fluorinated derivatives of norepinephrine (NE) has been continued. It has been shown that 5-fluoronorepinephrine (5-FNE), 6-FNE, and NE are equipotent as alpha-adrenergic agonists in the isolated guinea pig aortic strip while 2-FNE has only marginal activity. In the isolated guinea pig atria, 2-FNE and NE are equipotent as beta-adrenergic agonists, 5-FNE is approximately 7 times more potent, while 6-FNE is essentially inactive. Examination of the effects of 2-, 5-, and 6-FNE on cardiac contractural force ($\beta 1$), arterial blood pressure ($\beta 2$) and femoral blood flow (α) were compared to those of NE in the anesthetized dog: 2-FNE acts on $\beta 1$ and $\beta 2$-adrenergic receptors, 5-FNE on α and $\beta 1$ receptors, and 6-FNE only on α-adrenergic receptors.

Studies of the potential anti-leukemic histidine analog, 2-fluorohistidine (2-FH) have continued. It is now evident the 2-FH mimics its parent amino acid, histidine, with regard to incorporation into protein in vitro (cell-free protein synthesis) or in vivo (mouse-L-cells). The behavior of 2-FH, blocking incorporation of histidine into protein but not affecting leucine incorporation in cell free systems, can best be explained by its replacement of histidine in conversion to the amino-acylated tRNA and subsequent incorporation into protein. The latter is in agreement with earlier studies on the incorporation of 2-FH into TCA insoluble material in organ cultures of rat pineal glands and into murine proteins in vivo. Furthermore, polypeptide products synthesized in the Krebs II cell-free system in the presence of 2-FH appear to be identical in size and number to those synthesized in the presence of histidine.

Initial studies on the metabolic fate of [^{3}H]2-FH suggest the accumulation of a free metabolite in mouse tissue with an apparent half-life of 72 hours. Preliminary evidence indicates this metabolite to be 2-F-urocanic acid. In vitro studies have demonstrated the 2-F-urocanic acid is a powerful inhibitor of urocanase. A possible consequence of the formation of 2-F-urocanic acid in vivo may be a temporary interruption in the utilization of histidine as a one-carbon source for purine biosynthesis.

Proposed Course of the Project: The application of antibodies to COMT to the localization of the enzyme in brain and cardiovascular tissue will be continued; the role of COMT in neoplasms derived from ductal tissue of breast and other hormonally responsive tissue will be continued; the nature of the genetic differences in COMT levels in different rodent strains and in hybrid cell cultures will be continued. In view of the apparent importance of COMT in human mammary carcinomas, an examination for the presence of immunoreactive proteins derived from COMT in plasma from affected subjects will be undertaken. The unusual specificity conferred by the introduction of fluorine into the aromatic ring of NE may permit an investigation of the nature of specific receptor-agonist interactions. In order to pursue this investigation the application of several fluorine substituted amines becomes immediately apparent. Thus the α-methyl- and N-isopropyl derivatives have already been prepared. Further, the amino acid precursors, e.g. α-methyl-6-fluoro dopa, and α-methyl-2-fluorodopa are potentially of great clinical utility. The effects of these fluoro amino acid analogs on blood pressure responses in normotensive and hypertensive rats are in progress. It may be possible to deliver a specific alpha- or beta-adrenergic agonist via the respective 6- or 2-fluorodihydroxyphenylalanines, through the blood brain barrier, to centers in the brain. The amino acids will be investigated as substrates for dopa decarboxylase and the fluorodopamines will be investigated as substrates for dopamine-β-hydroxylase. Studies on the metabolism of 2-FH will be continued in order to confirm the identity of 2-fluorourocanic acid as the major metabolite. Studies are in progress to develop techniques and lipid soluble derivatives of 2-fluorourocanic acid to determine whether the antileukemic activity of 2-FH are related to the accumulation of 2-fluorourocanic acid in tissue.

Significance to Biomedical Research and the Program of the Institute: The biosynthesis, uptake, and metabolism of biogenic amines and their amino acid precursors are of fundamental importance in neurochemistry and pharmacology and have direct bearing on the function of both the central and peripheral nervous systems. The role of catechol-O-methyltransferase is important with regard to the functioning of the cardiovascular system, the detoxification of xenobiotic catechols, the inactivation of catechol steroids and in the metabolism of catecholamine transmitters in the central nervous system. An understanding of the role, localization, etc. of this enzyme may provide insight into a variety of pathological conditions in man, including essential hypertension and neoplastic disease of mammary ductal tissues. The unique agonist-specificities conferred by fluorine substitution on norepinephrine has provided a new and useful means of examining agonist-adrenergic receptor interaction and may lead to both a fundamental insight into the nature of such

receptors and provide pharmacological agents of clinical value. The studies on the mechanism of the antileukemic properties of 2-fluorohistidine may provide a new therapeutic approach to the control of leukemia.

Publications:

Kaplan, G. P., Hartman, B. K., and Creveling, C. R.: Immunohistochemical demonstration of catechol-O-methyltransferase in mammalian brain. Brain Res. 167: 241-250, 1979.

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Goldstein, D. J., Weinshilboum, R. M., Dunnette, J. H., and Creveling, C. R.: Developmental patterns of catechol-O-methyltransferase in genetically different rat strains: Enzymatic and immunochemical studies. J. Neurochem. 1979, in press.

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Usdin, E., Borchardt, R. T., and Creveling, C. R. (Eds.): Transmethylation, Developments in Neuroscience Vol. 5, New York, Elsevier/North-Holland, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 31102-09 LBC formerly Z01 AM 20001-08 LC

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Role of Cyclic Nucleotides in the Nervous System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. W. Daly, Chief, Laboratory of Bioorganic Chemistry, NIAMDD

Other:
C. R. Creveling, Research Chemist, LBC, NIAMDD
P. Skolnick, Pharmacologist, LBC, NIAMDD
C. W. Davis, Staff Fellow, LBC, NIAMDD
F. Smellie, Staff Fellow, LBC, NIAMDD
Y. Nimitkitpaisan, Staff Fellow, LBC, NIAMDD
M. Edwards, Chemist, LBC, NIAMDD
W. Padgett, Biologist, LBC, NIAMDD
E. McNeal, Biologist, LBC, NIAMDD
C. R. Partington, Guest Worker
F. Pons, Guest Worker
D. Atlas, Guest Worker
F. Bruns, Guest Worker

COOPERATING UNITS (if any)
J. N. Wells, Dept. Pharmacol., Vanderbilt Univ., Nashville, TN.; B. Hoffer, Dept. Pharmacol., Univ. Colorado, Denver; S. Snyder, Dept. Pharmacol., Johns Hopkins Univ., Baltimore, MD.

LAB/BRANCH
Laboratory of Bioorganic Chemistry

SECTION
Section on Pharmacodynamics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.3	6	0.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of cyclic nucleotide-generating systems to the functional operation of the central nervous system is being investigated from a number of standpoints: i) The nature of the control of adenylate cyclase or phosphodiesterase activity by neurotransmitters, guanyl nucleotides, gangliosides, phospholipids, calcium ions, monovalent cations, proteases, and other factors. ii) The development of selective inhibitors for the two major phosphodiesterases in rat brain, namely a calcium-dependent and a calcium-independent enzyme. iii) The nature of the activation of adenylate cyclase by a selective β-adrenergic agonist, 2-fluoronorepinephrine and a selective α-adrenergic agonist, 6-fluoronorepinephrine. iv) The nature of the facilitation of β-adrenergic, H_2-histaminergic, and adenosine-mediated activation of cyclic AMP systems in brain slices and brain vesicular preparations by α-adrenergic, H_1-histaminergic, and serotonergic agonists. v) The adenosine-dependent desensitization of receptor-adenylate cyclase complexes in brain vesicular preparations. vi) The relationship of membrane potential to cyclic AMP levels in brain vesicular preparations. vii) The factors involved in glutamate or depolarizing agent-elicited accumulations of cyclic AMP in brain slices.

Objectives: To determine the factors that govern the formation, metabolism, and action of cyclic AMP and cyclic GMP in the central nervous system. To investigate the possible role of cyclic nucleotides in regulating biochemical reactions and membrane properties in brain tissue. To investigate the interrelationship between central neurotransmitter function and cyclic nucleotides. To investigate the role of adaptive changes in cyclic nucleotide-generating systems in drug-tolerance and dependence. To investigate the role of membrane structure to the responsiveness of cyclic nucleotide-systems.

Methods Employed: Surgical techniques, environmental manipulation, drug-regimens have been used to alter central function. Formation and metabolism of cyclic nucleotides in biological preparations and with isolated enzymes have been studied with isotopically labeled compounds and with a variety of drugs and pharmacologically or biochemically-active agents.

Major Findings: Mixed brain gangliosides caused a 50-95% increase in the basal adenylate cyclase activity in membrane preparations from rat cerebral cortex. The activation was rapid and irreversible. The expression of activation showed a marked temperature dependence, with maximal effects seen in the 30-40° range and no activation seen at either 10° or 50°. The activation of adenylate cyclase by brain gangliosides was additive with respect to activation by fluoride and detergents, but was not additive with respect to activation by GppNHp or lysolecithin. The activation of the enzyme by gangliosides was unaffected by the presence of calcium ions, calcium-dependent activator protein, or EGTA. The addition of brain gangliosides to the membranes caused a consistent restoration of the responsiveness of the adenylate cyclase system to activation by β-adrenergic agents, an effect similar in magnitude to that observed by addition of guanyl nucleotides to the membrane preparations. (Partington)

Mixed brain gangliosides stimulated by about 1.5- to 2-fold the activity of rat cerebral cortical calcium-dependent and calcium-independent cyclic nucleotide phosphodiesterases in a concentration-dependent manner. The calcium-dependent enzyme was at least 10-times more sensitive to activation by gangliosides. Activation occurred rapidly and was readily reversible. Maximal activation of the calcium-dependent enzyme by gangliosides was only 50% of that elicited by lysolecithin or calcium-dependent activator protein. Activations by lysolecithin and gangliosides were apparently additive, while the activation by activator protein was slightly diminished in the presence of gangliosides. Trypsin-pretreatment of the calcium-dependent enzyme resulted in a loss of sensitivity to subsequent activation by gangliosides or activator protein without altering the activation by lysolecithin. Potassium ions inhibited basal activity of the calcium-dependent phosphodiesterase but had no effect on enzyme activated by gangliosides, activator protein, or lysolecithin. Phosphodiesterase in the membrane-bound state did not appear to be affected by gangliosides. It would appear that gangliosides may be involved in the regulation of both adenylate cyclase and phosphodiesterase in the central nervous system. (Davis)

Mild proteolysis of membrane preparations from rat cerebral cortex with low concentrations of endopeptidases such as trypsin or chymotrypsin caused a 50-400% increase in the basal adenylate cyclase activity. Maximal activation of adenylate cyclase was obtained by including the protease in the adenylate cyclase assay, although an activated preparation could be obtained by pretreatment of the membranes with chymotrypsin. The readily reversible activation of adenylate cyclase in membrane preparations by trypsin requires further investigation. The proteolytically activated enzyme retained responsiveness to activation by sodium fluoride and GppNHp, but was no longer activated by gangliosides or calcium dependent activator protein. The expression of activation of adenylate cyclase by protease treatment showed a marked temperature dependence, with maximal effects expressed in the 30-40° range and no activation expressed at either 10° or 50°. (Partington)

Incubation of rat cerebral cortical slices in Krebs-Ringer bicarbonate-glucose buffer induced a rapid decline in the responsiveness of the adenylate cyclase in subsequently prepared membrane preparations to stimulation by various activators of the enzyme. The desensitization of the adenylate cyclase system was time and temperature dependent, showed an absolute dependence on extracellular calcium ions, and was enhanced by the presence of serine proteases in the incubation medium, producing a membrane preparation which was partially responsive to activation by fluoride and GppNHp, but completely unresponsive to activation by ganglioside, proteolysis, or β-adrenergic agonists. The nature of the calcium-dependent mechanism involved in the loss of responsiveness of membranal adenylate cyclase is unknown, but it appeared that the loss of sensitivity might be the result of the action of an extracellular, membrane-bound, calcium-dependent protease. It is remarkable that in the slices prior to homogenization no evidence for such a time and calcium-dependent loss of responsiveness of cyclic AMP-generating systems is manifest. (Partington, Edwards)

Adenosine interacts with two classes of sites which affect adenylate cyclase: Extracellular R-sites through which the enzyme can be stimulated or inhibited and intracellular P-sites though which the enzyme is inhibited. In rodent brain slices, adenosine 5'-cyclopropylcarboxamide (EC_{50} 1 μM) is a potent agonist at a stimulatory R-site. Competitive antagonists versus adenosine (100 μM) at the R-site include 5'-methylthioadenosine (IC_{50} 100 μM), theophylline (IC_{50} 60 μM), and 8-phenyltheophylline (IC_{50} 6 μM). Adenine arabinoside and 2',5'-dideoxyadenosine are relatively potent agonists at the P-site. Specific binding of the radioactive adenosine carboxamide or of radioactive adenine arabinoside represents 25-45% of total binding to crude synaptosomal membranes from various brain regions. However, displacement profiles by various agonists/antagonists are not consonant with pure R- or P-site ligand properties for either the carboxamide or the arabinoside. The high affinity binding site for the arabinoside (Kd 170 nM) is present in excess over a high affinity site for the carboxamide (Kd 10 nM) in rat cerebral cortical membranes. (Nimitkitpaisan, Bruns, Pons, Smellie)

The stereoisomers of N_6-phenylisopropyladenosine elicit accumulations of cyclic AMP in brain slices via interaction with adenosine-receptors. The

response in guinea pig cerebral cortical slices and in rat hippocampal slices is blocked by theophylline, potentiated by biogenic amines and increased in the presence of a calcium chelator, EGTA. The l-isomer (EC_{50} 25 μM) is at least three-fold more potent than the d-isomer in eliciting accumulations of cyclic AMP in brain slices. In a rat coronal hippocampal slice in vitro, l-phenylisopropyladenosine reduces the amplitude of evoked synaptic responses generated via a monosynaptic pathway to the pyramidal neurons. The d-isomer is about 100-fold less potent. The difference in stereoselectivity with regard to activation of cyclic AMP-generating systems and with respect to inhibition of evoked synaptic potentials in hippocampal slices indicates that different adenosine-receptors are involved in these responses. (Smellie)

A series of 1,3-dialkylxanthines was examined as antagonists of adenosine-induced accumulation of cyclic AMP in guinea pig cerebral cortical slices and as inhibitors of brain phosphodiesterases. 8-Phenyltheophylline and 1,3-dibutylxanthine appear to represent potent adenosine-antagonists with relatively low activity as phosphodiesterase inhibitors whereas 7-benzyl isobutylmethylxanthine is a potent and relatively selective inhibitor of the calcium-dependent phosphodiesterase with little activity as an adenosine-antagonist. None of the compounds was a potent inhibitor of the brain calcium-independent phosphodiesterase, although 1-isoamyl-3-isobutylxanthine might prove useful as an inhibitor of this enzyme because of its very low activity as an adenosine-antagonist. Indeed, isoamylisobutylxanthine markedly potentiates adenosine-elicited accumulations of cyclic AMP in brain slices. The potency of other phosphodiesterase inhibitors on the two major brain enzymes indicated that a 3,4-dialkoxybenzyl-2-imidazolidinone, RO 201724, and a 3,4-dialkoxyphenyl-2-pyrrolidone, ZK 62771, represent relatively selective inhibitors of the calcium-independent enzyme. (Davis, Smellie)

A calcium-dependent cyclic nucleotide phosphodiesterase from rat cerebrum was, in the absence of activator protein, inhibited by various monovalent cations. The inhibition was rapid, readily reversible, and concentration-dependent with 100 nM potassium ions inhibiting essentially all basal enzyme activity, while 100 mM sodium ions produced only moderate inhibition. At 100 mM, potassium and sodium inhibited enzyme activated by the calcium-dependent activator by only 15 to 30%, while at 55 mM no inhibition pertained. Potassium and sodium ions at 55 mM had no effect on the calcium-independent phosphodiesterase from rat cerebrum. The results indicate that at normal intracellular concentrations of potassium ions the activity of the calcium-dependent phosphodiesterase is completely dependent on the presence of calcium plus activator protein. (Davis)

A rapid, simple, and direct assay for 3',5'-cyclic nucleotide phosphodiesterase activity is based on the effective separation of cyclic AMP, cyclic GMP, or cyclic CMP from their corresponding 5'-nucleotides and nucleosides by chromatography on a polyacrylamide-boronate gel. The affinity of the boronate residue for cis-diols results in the retention of 5'-nucleotides and nucleosides while 3',5'-cyclic nucleotides are not retained. The coelution of all 5'-nucleotides and nucleosides allows for the accurate assessment of phosphodiesterase activity in preparations contaminated by other purine me-

tabolizing enzymes such as 5'-nucleotidases and nucleotide and nucleoside deaminases. In most tissues nucleoside phosphorylase activity is very low and hence will not affect assays of phosphodiesterase activity based on use of these affinity gels. (Davis)

The accumulation of cyclic AMP elicited in guinea pig cerebral cortical slices by ATP or 5'-AMP is markedly antagonized by a combination of agents, $APCH_2P$ and GMP, which inhibit nucleotidase activity and by adenosine deaminase. Thus hydrolysis to adenosine appears requisite for the activation of cyclic AMP-systems in brain slices by adenine nucleotides. Only adenosine deaminase inhibits the accumulation of cyclic AMP elicited by depolarizing agents indicating that depolarizing agents cause primarily "release" of adenosine itself rather than ATP or other adenine nucleotide precursors of adenosine. (Pons, Bruns)

Glutamate at high concentrations elicits marked accumulations of cyclic AMP in guinea pig cerebral cortical slices. The glutamate-response is blocked completely by adenosine deaminase indicating a complete dependence on "release" of adenosine. The glutamate-response is not blocked by a specific glutamate antagonist, 1-hydroxy-3-aminopyrrolidone-2, indicating that it does not involve activation of the glutamate-receptors involved in the excitatory effects of glutamate on central neurons. Remarkably, the response to glutamate is potentiated by 2-chloroadenosine. It is proposed that glutamate causes both "release" of adenosine and other factors and that the latter potentiate the response of cyclic AMP-systems to "released" adenosine and to exogenous adenosine or 2-chloroadenosine. Such factors, like α-adrenergic agonists or H_1-histaminergic agonists are proposed to have no direct effect on cyclic AMP-systems. (Pons, Bruns)

Norepinephrine and 5-fluoronorepinephrine elicit a 15-fold accumulation of radioactive cyclic AMP in adenine-labeled rat cerebral cortical slices. The response to those agonists is partially blocked by either α- or β-antagonists, indicating a mixed α- and β-adrenergic activity for both compounds. Isoproterenol and 2-fluoronorepinephrine elicit an 8-fold accumulation of cyclic AMP which is antagonized by β-adrenergic blocking agents but not by α-adrenergic blocking agents. Responses to isoproterenol and 2-fluoronorepinephrine are not additive and appear to involve β_1-adrenergic receptors in rat cerebral cortex. 6-Fluoronorepinephrine elicits a 3-fold accumulation of cyclic AMP which is antagonized significantly by α-adrenergic blocking agents but not by β-adrenergic blocking agents. The response to 6-fluoronorepinephrine and the α-component of the norepinephrine-response shows certain of the characteristics of α_1-adrenergic receptors. Combinations of 6-fluoronorepinephrine with adenosine, with isoproterenol, or with 2-fluoronorepinephrine have greater than additive effects on cyclic AMP levels in rat cerebral cortical slices. The response to 6-fluoronorepinephrine is not significant if both adenosine- and β-receptors are blocked by the presence of 8-phenyltheophylline and propranolol. In guinea pig cerebral cortical slices, the norepinephrine derivatives have marginal effects on cyclic AMP levels. However, adenosine-elicited or histamine-elicited accumulations of cyclic AMP in guinea pig cerebral cortical slices are markedly potentiated by norepinephrine, 5-fluoro-

norepinephrine, and 6-fluoronorepinephrine but not by the β-adrenergic agonists, isoproterenol, or 2-fluoronorepinephrine. It is proposed that α-adrenergic agonists affect cyclic AMP-generating systems in brain slices solely by potentiation of the activation of such systems by adenosine, β-adrenergic, or histaminergic agonists. (Padgett, Creveling)

Binding of radioactive dihydroalprenolol to β_1-adrenergic receptors in rat cerebral cortical membranes is antagonized by norepinephrine, 5-fluoronorepinephrine and 2-fluoronorepinephrine. The 6-fluoro-analog of norepinephrine is at least 100-fold less potent. In contrast, binding of radioactive WB-4101 to α_1-adrenergic receptors in rat cerebral cortical membranes is antagonized by norepinephrine, 5-fluoronorepinephrine and 6-fluoronorepinephrine with 2-fluoronorepinephrine being 100-fold less potent. No such remarkable effects of the position of fluorine-substitution on the affinity of dopamines for β-adrenergic, α-adrenergic, or dopaminergic receptors were noted. Thus, it appears likely that interactions between the benzylic hydroxyl and the 2-fluoro or 6-fluoro substituent are responsible for the lack of α-adrenergic or β-adrenergic activity, respectively in these compounds. (Nimitkitpaisan)

Norepinephrine, histamine, adenosine, glutamate, and depolarizing agents elicit accumulations of radioactive cyclic AMP from adenine-labeled nucleotides in particulate fractions from Krebs-Ringer homogenates of guinea pig cerebral cortex. The particulate fractions contain vesicular entities associated with a significant portion of the membranal adenylate cyclase. Particulate fractions from sucrose homogenates are a less effective source of such responsive entities. The responses to various agents are quite similar to responses which pertain with brain slices. Activation by norepinephrine is via α-adrenergic receptors, while activation by histamine is via H_1- and H_2-histaminergic receptors. Adenosine responses are potentiated by the amines and are antagonized by alkylxanthines. Glutamate and depolarizing agents elicit accumulations of cyclic AMP via "release" of endogenous adenosine. It is proposed, based on the absence of an α-adrenergic or H_1-histaminergic response in the presence of a combination of potent adenosine and H_2-histaminergic antagonists, that α-adrenergic and H_1-histaminergic receptor mechanisms do not activate adenylate cyclase directly in Krebs-Ringer particulate fractions, but merely facilitate activation by β-adrenergic, H_2-histaminergic, or adenosine-receptors. (McNeal, Creveling, Partington)

The accumulations of radioactive cyclic AMP elicited by adenosine, norepinephrine, and histamine in adenine-labeled vesicular entities of a particulate fraction from guinea pig cerebral cortex are greatly reduced as a result of prolonged preincubation. The presence of adenosine deaminase during preincubations largely prevents the loss of adenosine, norepinephrine, and histamine-responses. Adenosine deaminase was inactivated by deoxycoformycin prior to stimulation of cyclic AMP-accumulation by adenosine or amines. Adenosine deaminase cannot restore responsiveness of the cyclic AMP-generating systems. It is proposed that in particulate fractions of guinea pig cerebral cortex, low levels of adenosine cause a slow loss of both adenosine- and amine-receptors and/or of coupling of receptors to cyclic AMP-generating systems. (McNeal, Creveling)

Membrane potential of vesicular entities in Krebs-Ringer particulate preparations was assessed by measuring the rate of influx of radioactive methyltriphenyl phosphonium. Membrane depolarization by potassium ions caused the largest reduction in rate of influx, while selective neuronal depolarization by veratridine or batrachotoxin caused a lesser reduction. Ouabain also caused a reduction. Neither excitatory nor inhibitory neurotransmitters had any effect, nor did neurotransmitter-elicited accumulation of cyclic AMP affect the rate of influx of the phosphonium compound into the vesicular entities. (McNeal, Creveling)

Significance to Biomedical Research and the Program of the Institute: The key role of cyclases and cyclic AMP and cyclic GMP in regulating cellular activity in response to external stimuli in many biological systems make elucidation of their role in brain of fundamental importance to an understanding of function in this organ and to the development of drugs for the treatment of malfunctions involving cyclic nucleotide systems.

Proposed Course of Project: The physiological significance of calcium-dependent activator protein, phospholipids, gangliosides, guanyl nucleotides, and proteases for the regulation of adenylate cyclases and phosphodiesterases will be investigated. The morphological location of the labeled pools of adenine nucleotides will be determined. The role of phosphoribosyl transferase and adenosine kinase in maintaining this pool will be explored. Secondary effects on enzymes and modulators due to elevated cyclic AMP levels in brain tissue will be investigated. The in vivo effects of drugs and the effects of lesions and hormonal or dietary alterations on the responsiveness of the cyclic AMP-systems will be further explored. The nature of the adenosine-receptor will be studies further using adenosine analogs and antagonists modified for possible affinity labeling. Other agents with selective effects on adenosine, adrenergic, serotonergic, and histaminergic systems will be sought and investigated. Fluorescent and photo-affinity ligands for a variety of receptors will be investigated. The role of calcium ions and its interrelationship with prostaglandin and adenosine in the function of cyclic AMP-generating systems will be further investigated, both in vivo and in vitro. Correlations between cyclase responses and behavioral activity will be studied in crosses between various rodent strains. The nature of the GTP-binding site on adenylate cyclase will be investigated. Correlations between cyclic GMP and cyclic AMP levels in brain slices will be attempted. Antibodies to enzymes and regulatory modulator proteins involved in the cyclic AMP-generating systems will be prepared and effects of various regimens on the turnover, levels, and distribution of these enzymes and regulatory proteins in brain will be investigated. Further attempts to develop more selective inhibitors of cyclases, phosphodiesterases, and kinases will be made. The mechanism of amine-adenosine interactions and desensitization of receptor-adenylate cyclase complexes will be probed in slices and vesicular entities from brain. Correlations of cyclic AMP-levels, membrane potential, and protein phosphorylation will be investigated further.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 31103-01 LBC

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Role of Benzodiazepine Receptors in the Nervous System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Phil Skolnick, Pharmacologist, Section on Pharmacodynamics, LBC, NIAMDD

Evan Williams, Visiting Fellow, LBC, NIAMDD
Kwan-Ling Lock, Guest Worker, LBC, NIAMDD
Ryotaro Tsujimura, Guest Worker, LBC, NIAMDD

COOPERATING UNITS (if any) S. Paul, CPB, NIMH; P. J. Marangos, CPB, NIMH; J. Tallman, APB, NIMH; J. Barker, LNP, NINCDS; J. F. MacDonald, LNP, NINCDS; M. Zatz, LCS, NIMH; K. Rice, LC, NIAMDD; D. Woodward, Dept. Physiology, Univ. Texas; D. Johnson,

LAB/BRANCH
Laboratory of Bioorganic Chemistry

SECTION
Section on Pharmacodynamics

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland, 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
The identification of saturable, high affinity, and stereospecific binding sites for benzodiazepines has been described in the mammalian central nervous system. The correlations between the binding of a series of benzodiazepines in vitro and their pharmacologic potencies as anxiolytics, anticonvulsants, and muscle relaxants in vivo, strongly suggest these sites are receptors mediating the therapeutic actions of the benzodiazepines. The physiologic role(s) of the benzodiazepine receptor and the pharmacologic, behavioral, and physiologic regulation of this receptor are being investigated using several experimental paradigms: i) the isolation and identification of endogenous substrates which subserve this receptor. ii) pharmacologic assessment of endogenous substances isolated and identified as having affinity for the benzodiazepine receptor in in vitro tests. iii) development of suitable in vitro models for studying the benzodiazepine receptor such as primary and transformed cultures of neural origin and mammalian retina. iv) development of novel benzodiazepines to characterize receptor subgroups and to determine the physiologic function of these subgroups.

PHS-6040
(Rev. 10-76)

Cooperating Units (Cont'd.): Dept. Pharmacol., Univ. Saskatchewan; P. J. Syapin, Dept. Neurology, Univ. So. California; E. Majchrowicz, LPS, NIAAA.

Project Description:

Objectives: To characterize and assess the physiologic role(s) of the benzodiazepine receptor in the mammalian central nervous system and to develop suitable in vitro model systems. To isolate, identify, and evaluate substances isolated from mammalian brain which may function as endogenous substrates (ligands) to this receptor. To develop novel compounds useful in characterization of these receptors and define the physiologic role and pharmacologic function of the receptor. To assess the importance of the benzodiazepine receptor in adaptive changes following physiologic, pharmacologic, and/or behavioral changes.

Methods Employed: Neurochemical characteristics of the benzodiazepine receptor have been studied with a radioreceptor assay using tritium labeled benzodiazepines. Isolation and identification of endogenous substrates for this receptor have utilized column chromatography (gel filtration and ion-exchange), high pressure liquid chromatography, thin layer chromatography, and biochemical techniques. Pharmacologic testing of these ligands was accomplished using intraventricular injection and peripheral injection of standard convulsants. Environmental manipulation, electroconvulsive shock and chemical intervention in vitro have been used to determine the importance of the receptor in maintaining physiologic and behavioral homeostasis and in determining the pharmacologic importance of these receptors.

Major Findings: Inosine and hypoxanthine have been identified as endogenous compounds capable of altering benzodiazepine binding to receptors in vitro. Inosine and hypoxanthine compete for benzodiazepine binding sites at "brain specific" receptors but have no affinity for "kidney" (peripheral) receptors. Furthermore, both inosine and hypoxanthine cross-react with a diazepam (Valium)-specific antibody, suggesting a common recognition site by the immunologic system. Other purines, such as caffeine, also bind to the benzodiazepine receptor and these findings may represent a pharmacologic mechanism for the anxiogenic and convulsant properties of caffeine. The classic convulsant pentylenetetrazole was also found to interact with the benzodiazepine receptor at concentrations which are attained following a convulsant dose of this drug.

Iontophoretic application of inosine to cultures of spinal cord obtained from fetal mice results in a dual action: The first is a rapid, depolarizing response which is also elicited by a water-soluble benzodiazepine, flurazepam. Inosine and flurazepam display cross-desensitization of this response, suggesting a similar agonist action at a receptor level. The second action of inosine is a slow hyperpolarization which is antagonized by flurazepam.

The importance of benzodiazepine receptors in mediating pentylenetetrazole induced convulsions was examined in mice. An excellent correlation was demonstrated between the number of "diazepam-occupied receptors" following

a standard anticonvulsant dose of diazepam and the number of animals protected against convulsions by a standard convulsant dose of pentylenetetrazole. Furthermore, no more than thirty percent of receptors need be occupied to fully manifest anticonvulsant activity, suggesting the IC_{50} or Ki of a drug or endogenous ligand may not be a relevant measure of the ability of a compound to exert a pharmacologic or physiologic effect via the benzodiazepine receptor.

The role of the benzodiazepine receptor in mediating seizure activity was studied using two seizure models: pentylenetetrazole-induced convulsions and electroshock. A rapid increase in the number, but not affinity of benzodiazepine receptors was observed following tonic-clonic convulsions induced in both these models. This increase was fully manifest 15 minutes post-ictal and was no longer apparent 60 minutes post-ictal.

Binding of radioactive benzodiazepines to brain receptors was demonstrated in vivo. This was the first report of stereospecific, organ specific binding in vivo. The regional distribution of benzodiazepine binding in vivo showed a perfect rank-order correlation with binding obtained in vitro. This technique may be of use in demonstrating drug/behavioral induced changes in the benzodiazepine receptor in vivo.

Intraventicular administration of inosine to mice afforded a partial protection from pentylenetetrazole-induced convulsions. This protection was expressed as an increase in the latency to convulsions following pentylenetetrazole rather than a reversal. Pharmacokinetic data supports the hypothesis that the transient action of inosine is due to a rapid metabolism and transport of this purine from the brain following intraventicular injection. Intraventricular administration of 2'-deoxyinosine, which is more potent than inosine in binding to benzodiazepine receptors resulted in a marked increase in latency of convulsions, while administration of 7-methylinosine and thymidine, both ineffective in displacing benzodiazepine binding in vitro, resulted in no alterations in latency. These findings support a benzodiazepine-like action of inosine, analogous to that which might be expected of an "ultrashort-acting benzodiazepine".

A simple and sensitive radioreceptor assay for benzodiazepines in plasma and other biological tissues was developed. The sensitivity obtained is equal to or greater than currently used technique of gas-chromatography with electron capture detection. In addition, the radioreceptor technique is rapid, requires no expensive equipment other than that available in most clinical laboratories, and gives a measure of the therapeutic equivalents of benzodiazepine which can be compared directly to diazepam. Patent rights for this technique are pending (issued to Drs. Skolnick and Paul through NIH).

Various models for studying the benzodiazepine receptor have been investigated, including mammalian retina, transformed cells of neural origin, and primary cultures of fetal brain and spinal cord. Data obtained from these models suggests that both the retina and primary cultures of brain and spinal cord may be used to study "brain specific" receptors in culture in order to

examine the physiologic and pharmacologic function(s) of this receptor.

Irazepine, a noncompetitive, irreversible inhibitor of benzodiazepine binding is the first in a series of new synthetic compounds which will be utilized to investigate the structure and biochemical characteristics of the receptor.

The effects of alcohol dependence and withdrawal from alcohol on the benzodiazepine receptor have been examined. Small but significant reductions in benzodiazepine receptor number have been demonstrated in animals undergoing alcohol induced convulsions. (Williams)

Isolation of two substances from bovine brain and pituitary which inhibit diazepam binding to brain tissue in vitro has been accomplished. These compounds were not isolated from bovine liver or adrenal, suggesting a specificity to the central nervous system. These compounds are higher molecular weight than inosine or hypoxanthine; one component is only partially dialyzable, suggesting a molecular weight of 1000-5000 daltons.

Significance to Biomedical Research and the Program of the Institute: The benzodiazepines are the most widely prescribed drugs in current therapeutic use. Understanding their mechanism of action and identification of endogenous compounds which may function as "benzodiazepine-like" compounds is of fundamental importance to the understanding of diseases such as epilepsy, anxiety-neuroses, and alcoholism, as well as development of a more fundamental understanding of brain function. In addition, these studies may provide information regarding the development of therapeutic agents superior to the benzodiazepines.

Proposed Course of Project: The importance of purines in various disease models (e.g. epilepsy) will be studied. Alterations in benzodiazepine receptors following two seizure models pentylenetetrazole and electroconvulsive shock will be expanded to determine if interaction with the benzodiazepine receptor is a universal first step in the generation and prevention of seizures. The structural requirements for purines to bind to the benzodiazepine receptor will be studied. The alterations in benzodiazepine receptors following changes in chemical, pharmacologic, or physiologic environment in cultured models will be examined. Irreversible inhibitors of benzodiazepine receptors will be used to attempt to measure receptor turnover in vivo, isolate the benzodiazepine receptor, and characterize receptor subtypes. The identification of brain and pituitary inhibitors of benzodiazepine binding will be attempted.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 31104-11 LBC formerly Z01 AM 20200-10 LC

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. M. Jerina	Chief, Sect. on Oxidation Mech.	LBC	NIAMDD
OTHER:	H. Yagi	Visiting Scientist	LBC	NIAMDD
	D. R. Thakker	Senior Staff Fellow	LBC	NIAMDD
	R. N. Armstrong	Staff Fellow	LBC	NIAMDD
	D. R. Buhler	Guest Worker	LBC	NIAMDD
	M. Tada	Guest Worker	LBC	NIAMDD
	B. Kedzierski	Guest Worker	LBC	NIAMDD
	Y. Ittah	Visiting Fellow	LBC	NIAMDD
	M. Nordqvist	Visiting Fellow	LBC	NIAMDD

COOPERATING UNITS (if any)

Drs. A. Conney, W. Levin and A. Wood, Hoffmann-La Roche, NJ; Dr. D. Whalen, Department of Chemistry, University of Maryland at Catonsville, Baltimore, MD; Dr. D. R. Boyd, Department of Chemistry, Queen's University, Belfast, N. Ireland

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7	6	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The primary goal of this project has been the elucidation of the structure of reactive metabolites which are responsible for the carcinogenic, cytotoxic, and mutagenic activity of benzo[a]pyrene and other polycyclic hydrocarbons. The approach taken consists of: i) synthesis of primary oxidative metabolites as well as selected secondary oxidative metabolites, ii) study of the metabolism of these hydrocarbons with liver microsomes, as well as with purified and reconstituted cytochrome P-450 systems with and without epoxide hydrase, iii) tests for inherent mutagenicity of the synthetic metabolites toward bacterial and mammalian cells, iv) elucidation of the roles of the cytochrome P-450 system and epoxide hydrase in potentiating or obliterating the mutagenicity of these metabolites, v) determination of the carcinogenic activity *in vivo* for these compounds, vi) determination of the rate and nature of the products formed when reactive metabolites such as arene oxides and diol epoxides react with biopolymers and less complex model compounds. A general theory of hydrocarbon-induced carcinogenesis has been formulated and its predictions are being tested with synthetic potential metabolites of several hydrocarbons. Based on the presently available data, the bay region theory has excellent predictive value.

Project Description:

Objectives: To study the basic mechanism of enzymatic oxygen activation and incorporation into organic substrates. To explore enzymatic and nonenzymatic reactions of labile intermediates such as epoxides and peroxides. To devise methods for the synthesis and study of the chemical reactions of arene oxides. To investigate the role of labile oxygenated intermediates in the mutagenicity, cytotoxicity, and carcinogenicity of xenobiotic substances.

Methods Employed: Isotopically labeled substrates, water, and oxygen gas have been used to study the oxidation of organic compounds to labile metabolites by a variety of enzymes. Nuclear magnetic resonance spectroscopy, mass spectrometry, x-ray crystallography and other physico-chemical techniques have been employed frequently in these studies. In addition to the usual techniques employed for the separation of metabolites, high pressure liquid chromatography is being pioneered in this area. Mutagenesis is studied in bacterial and mammalian cells and correlated with whole animal carcinogenicity.

Major Findings: Previous annual reports from this Section have described a systematic approach to the study of the cytotoxicity, mutagenicity, and carcinogenicity of the ubiquitous environmental carcinogen benzo[a]pyrene (BP). Briefly these studies have consisted of i) synthesis of as many known and potential oxidative metabolites as was possible, ii) study of the metabolism of the hydrocarbon with these authentic standards in hand, iii) testing of these compounds for cytotoxic and mutagenic activity with bacterial and mammalian cells both in the presence and in the absence of added drug metabolizing systems such as cytochrome P-450 and epoxide hydrase, and iv) evaluation of the carcinogenicity of these synthetic standards in several animal models. These studies provided evidence which indicated that (+)-7β,8α-hydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo-[a]pyrene is the most important carcinogenic metabolite of benzo[a]pyrene. With the first identification of an ultimate carcinogenic form for any member of the polycyclic aromatic hydrocarbon class of carcinogens, we formulated the "bay-region" theory which predicts that diol epoxides that have the epoxide group in the bay-region of the hydrocarbon will be the most chemically reactive and presumably biologically active. To test the predictions of the theory, we have continued studies on the hydrocarbons benzo[a]anthracene, chrysene, dibenzo[a,h]anthracene, and 3-methylcholanthrene which were initiated in the previous year.

Benzo(a)pyrene (BP). In the previous report we described tumor studies which established that the metabolic pathway responsible for the major part of the carcinogenicity of BP consisted of i) initial formation of BP 7,8-oxide by the cytochrome P-450 system, ii) hydration to BP 7,8-dihydrodiol by epoxide hydrase, and iii) cytochrome P-450 catalyzed formation of 7,8-diol-9,10-epoxides. Initial tumor studies on mouse skin indicated that the (-)-7,8-dihydrodiol was more active than its (+)-enantiomer For this reason, the role of absolute stereochemistry in modulating tumorigenicity

has been explored further with the newborn mouse tumor model. In this model, the (-)-diol is 16 to 60-fold more active than the (+)-enantiomer and induces lymphoma as well as lung adenomes. When the four metabolically possible enantiomeric diastereomers of the 7,8-diol-9,10-epoxides were examined in this model, only the (+)-7β,8α-diol-9α,10α-epoxide showed marked tumorigenicity. High activity for this isomer only had been predicted by mutagenicity tests with Chinese hamster V79 cells whereas the S. typhimurium tester strains had predicted high activity for all four isomers. The exceptional activity of the one isomer dramatically points out the importance of absolute stereochemistry, to carcinogenesis, a factor which was overlooked in the past. We have also synthesized (+)- and (-)-BP 7,8-oxide to test them in the newborn mouse model. BP 7,8-oxide is less active than BP in this tumor model as it was on mouse skin.

On oral administration, butylated hydroxyanisole was found to inhibit the carcinogenicity of both BP and BP 7,8-dihydrodiol. These results suggest that the inhibiting effect entails events which occur subsequent to the formation of the diol. The BP 7,8-diol-9,10-epoxides elicit damage to DNA which is recognizable by the ultraviolet excision repair system in normal human cells.

Benzo(a)anthracene (BA). Previous studies from our laboratories had shown that BA 3,4-dihydrodiol and 3,4-diol-1,2-epoxides were highly tumorigenic in accord with the predictions of the bay-region theory. However, other laboratories had claimed that the 3,4-dihydrodiol was not a metabolite of BA. Such has not proved to be the case. Careful chromatography of the liver microsomal metabolites of BA has established that the 3,4-dihydrodiol accounts for as much as 4% of the total metabolism. The fact that the amount of this metabolite is so small partially explains why BA is a somewhat weaker carcinogen than might have otherwise been expected.

Dibenzo(a,h)anthracene (DBA). The bay region theory predicts that the 3,4-dihydrodiol of DBA should be a proximate carcinogen of DBA. In initiation-promotion experiments on mouse skin (10 nmol), this dihydrodiol was found to be twice as active as DBA whereas the 1,2- and 5,6-dihydrodiols were inactive in causing the formation of papillomas. Results of tumorigenicity tests of these compounds in the newborn mouse model provided similar results. One particularly interesting aspect of these studies is the fact that the 5,6-dihydrodiol is a compound which acts as a false positive in the Ames Test since this compound can be activated to mutagens but is not carcinogenic.

3-Methylcholanthrene (MC). The 9,10-dihydrodiol of MC is predicted to be a proximate carcinogen of this hydrocarbon. Previously we reported that we were unable to detect this compound as a metabolite of MC. We were, however, able to isolate two diastereomeric 9,10-dihydrodiols of 1-hydroxy MC. One of these has proved to be from 5 to 100-fold more mutagenic to S. typhimurium strain TA 100 and to Chinese hamster V79 cells on metabolic activation than was MC. Since we pointed out the difficulty in isolating dihydrodiols from methylated hydrocarbons, other laboratories have sought

and found hydroxymethylated dihydrodiols from 7-methyl BA and 7,12-dimethyl BA. These hydroxy-dihydrodiols are also thought to be proximate carcinogens.

Physical Organic and Synthetic Aspects of Drug Metabolism. In a continuing effort to relate chemical reactivity to biological activity, we have studied the hydrolysis of bay region diol epoxides of phenanthrene and chrysene in order to compare their reactivities to that of the corresponding BP derivatives. In all three cases, the diol epoxide-2 isomer with the benzylic hydroxyl group trans to the oxirane ring has proved more reactive in acid. In contrast, the diol epoxide-1 diastereomers are more reactive in the neutral to basic pH range possibly due to anchimeric assistance. Since tetrahydroepoxides are more reactive than their corresponding diol epoxides, conformation factors were suggested as major reasons for the differences in rate.

Benzo[e]pyrene is predicted to have more reactive diol epoxides than chrysene yet is inactive as a carcinogen. For this reason we have synthesized the two metabolically probable dihydrodiols of B[e]P in order to explore in detail this apparent exception to the bay-region theory.

Drug Metabolizing Enzymes. Studies have continued on the enzyme epoxide hydrase. Immunoprecipitated enzyme with antibody from four different sources retains full catalytic activity. Cross-reactivity studies have indicated that different species contain different forms of the enzyme. At present, it is still not possible to establish whether the highly purified preparation from rat liver constitutes a single polypeptide chain.

Significance to Biomedical Research and the Program of the Institute: Although enzymatic oxidation and drug metabolism has long been held to constitute a means of detoxication, results of the type described here make it quite clear that certain of these pathways for drugs and environmental chemicals result in toxic and carcinogenic metabolites. Once the nature of these active metabolites has been identified, it may become possible to predict which industrial and agricultural chemicals are unsafe for use. The potential impact on the design of safe drugs is clear. A workable theory of hydrocarbon-induced carcinogenicity with predictive properties is of immense practical value.

Proposed Course of Project: The initial success of the "Bay-Region Theory" demands that this potentially powerful concept be examined in the fullest. To this end, we propose the synthesis of dihydrodiols and diol epoxides from other carcinogenic and noncarcinogenic hydrocarbons. Carcinogenesis testing and metabolism studies will be continued. The mechanism of action of the cytochrome P-450 system and epoxide hydrase will be further explored.

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Annual Report of the

Mathematical Research Branch

National Institute of Arthritis, Metabolism, and Digestive Diseases

If A is the system-matrix for a linear system, then with output which is a linear combination (coefficients $c_1, c_2, \ldots, c_n$) of the state variables, the matrix governing output-feedback control is $A + bc^*$ where c is the vector of the c_i and b the control vector. A famous theorem asserts that if the original system is state controllable then there exists a c such that $A + bc^*$ has arbitrary spectrum. We have discovered that there exists in the literature no legitimate coordinate-free proof of this theorem. We have given a correct coordinate-free proof based in part on our previous work on control theory (J. Res. Nat. Bur. Stds. 82, 129, 1977). The result is stated as a general theorem on the spectrum of a rank-one perturbation of a nonderogatory matrix. The control-theoretic version is a special case. (Dr. J. Z. Hearon).

Previously (Math. Biosci. 15, 69, 1972) we have derived a general expression for the mean residence time in a compartmental system under arbitrary initial conditions. This has now been extended to arbitrary inputs. In both cases the distribution of residence times and the moments of the distributions have been given in a form feasible for compution from powers of the inverse system-matrix alone (Dr. J. Z. Hearon).

A numerical method to solve for the facilitated transport of oxygen in the presence of hemoglobin or myoglobin was reported previously. With this method an extensive analysis of the available experimental information has been carried out. From the results obtained one can conclude that the observed facilitation can be accounted for by the simultaneous diffusion of oxygen, the protein and the oxygen-protein compound plus their chemical interaction. A paper containing these results has been submitted for publication. In the striated muscles there exist membranes, the sarcoplasmic reticulum and the muscle fiber membrane, that may impede the diffusion of the protein carrier. A theoretical study was undertaken to analyze the facilitated transport in the presence of membranes on the diffusion path. By using the spatial parameters and the chemical kinetics appropriate to the striated muscle a number of computations were carried out. The results indicate that the presence of those membranes may not interfere significantly with the facilitated transport in vivo. A paper with these results has been submitted for publication. (Dr. J. M. Gonzalez-Fernandez and Mrs. S. E. Atta).

Neural modeling that was originally directed at understanding certain experimental observations in photoreceptors of a nudibranch mollusc, has led to theoretical results and insights which have application to other nervous systems and to observations related to conditioning phenomena not only in insects and mollusks, but also in the mammalian central nervous system. Computations were aimed at distinguishing between different aspects of the long lasting depolarization (LLD) observed by Alkon and Grossman following light input to these molluscan photoreceptors. Their experiments showed that this LLD involves not only significant membrane depolarization of the photoreceptor soma, but also a very significant increase of soma membrane resistance. Our

modeling enabled us to separate the effects of the resistance increase from the effects of the depolarization. We could demonstrate (both analytically and computationally) some important consequences of the membrane resistance increase. Briefly, the increase of soma membrane resistance not only increases the input resistance of the neuron measured either at the soma or in the dendrites, it also significantly increases the slowest membrane time constant, τ_0, of the model system. Although the increased input resistance did result in an expected increase of synaptic potential amplitudes when the synaptic activation had a slow time course, this effect was negligible for brief synaptic potentials. However, we predicted and then demonstrated computationally that the increase of τ_0 is responsible for a prolonged decay time and for a significant increase in the effectiveness of temporal summation of brief synaptic potentials (in both regular and stochastic temporal sequences). Now that this contribution to increased depolarization (by temporal summation of EPSP) has been demonstrated and understood theoretically, its contribution to the complicated associative behavioral changes observed by Alkon and colleagues can be explored by future modeling. Also, this effect seems likely to be relevant to cases of increased resistance observed following conditioning of neurons in the locust (Hoyle and colleagues at the Univ. of Oregon) and in the cerebral cortex of cat (Woody and colleagues at UCLA). [W. Rall collaborating with Drs. J. Rinzel and R. Miller, and with Dr. D. L. Alkon of NINCDS].

Detailed illustrative computations have been carried out and presented to biologist audiences to show them how the mathematical treatment of dendritic branching (Rall, 1959) can be applied to the wealth of new data now being obtained by many investigators. The extensive branching of individual neurons from many electrophysiological preparations can be revealed and measured with the help of Lucifer yellow, horseradish peroxidase, or cobalt with intensification by silver. Functional interpretation of such branching depends upon such biophysical computations. (Dr. W. Rall).

Work has continued on epithelial transport. Reports on earlier work are in press (Huss, R. E., and Stephenson, J. L. A mathematical model of proximal tubule absorption. J. Membrane Biology; Weinstein, A. M. and Stephenson, J. L. Electrolyte transport across a simple epithelium: Steady state and transient analysis. Biophysical Journal.) Recent attention has been focused on solute-linked water transport in a leaky epithelium. The analytical model of the lateral intercellular space has been developed and used to identify the determinants of isotonic transport, to estimate the influence of intraepithelial solute polarization on the measurement of epithelial water permeability, and to discuss the capability of interspace models to simulate transport of water against an osmotic gradient. For a compliant interspace, the analytical model has been used to isolate the time constants of intraepithelial transients in a form suitable for experimental investigation. Analytical predictions have been verified against the more complete numerical epithelial models.

A collaborative project with the Laboratory of Kidney and Electrolyte Metabolism, NHLBI, has involved the simulation of passive transport of CO_2 across proximal tubule in an isolated perfused tubule preparation. The effect of intratubular buffering on the steady state CO_2 concentration profile has

been estimated analytically. A numerical model that defines the collected fluid pH as a function of perfusion conditions and tubule permeability has been used in the reduction of experimental data. (Weinstein, A. M. and Stephenson, J. L.).

Improved numerical methods were developed and implemented for solution of the kidney equations. A rank one update method for improving the inverse of a Jacobian matrix has resulted in a reduction in computational work from $O(n^3/3 + n^2)$ to $O(n^2)$, where $n \approx 70$ variables for some models of interest. The analytic expression of the inverse of the pointwise Jacobian matrix has been extended using the algebraic manipulator MACSYMA to include as many as $M = 6$ unknowns (hydraulic pressure, four solutes, and volume flow) with a concomitant reduction in work from $O(m^3/3 + m^2)$ to $O(m^2)$ (Mejia, R. and Stephenson, J. L. Symbolics and numerics of a multinephron kidney model. Proc. 1979 MACSYMA User's Conference, Washington, D. C.)

These and other improved numerical methods have been implemented in a full kidney multinephron model (Mejia, R. and Stephenson, J. L. Numerical solution of multinephron kidney equations. J. Comput. Physics, in press), and a multinephron central core model as well as a single nephron vasa recta model. Data previously published have been verified with these models.

Work has continued on the qualitative analysis of renal flow networks. An analysis of the system of equations describing pressure driven flow through a tube in a bath has been completed and submitted for publication. An analysis and numerical study of a three tube central core model of the renal medulla was given. The study demonstrates the existence of several solutions to the equations of the model, and discusses their behavior as a function of inlet flow and water permeability (Dr. J. Garner, Dr. B. Kellogg and Dr. J. Stephenson).

Theoretical results for the Hodgkin-Huxley (HH) equations for space-clamped squid axon demonstrate that for a range of just suprathreshold current step intensities there are two stable states, one oscillatory (repetitive firing) and one steady (maintained depolarization). This multiplicity, which implies hysteresis and switching phenomena, motivated an experimental-theoretical collaboration with R. Guttman and S. Lewis (Woods Hole, MBL). The experiments, carried out for squid axon bathed in low calcium, showed that repetitive firing in response to a just suprathreshold step can be annihilated by a brief depolarizing or hyperpolarizing pulse of the proper magnitude applied at the proper phase. Moreover, hysteresis was demonstrated directly by using a slow up and then down current ramp. Physiological implications include stabilization against noise for near threshold behavior and a possible mechanism to start or stop a biological pacemaker with a brief synaptic input. These results were presented at the Biophysical Society Annual Meeting (February, 1979) and at the Gordon Research Conference on Theoretical Biology and Biomathematics (June, 1979); a manuscript has been submitted to J. Physiology.

Additional calculations with R. Miller for these HH equations at 6.3°C have revealed periodic solutions of an even greater variety than previously obtained or conjectured. The calculations suggest that these solutions which are unstable arise through a cascade of bifurcations to limit cycles of successively doubled periods. These along with our previously obtained results on periodic solutions to the HH equations are included in a manuscript (soon

to be submitted) which also describes in detail our numerical method to obtain periodic solutions (stable or unstable). We expect this methodology to be useful for a variety of nonlinear oscillation problems.

Nerve membrane response to sinusoidal applied current, as well as steady current (as above), has been under study. Specific examples for the HH equations and FitzHugh-Nagumo equation demonstrate that for some stimulus parameters there may be more than one stable phase-locking response. In contrast, the integrate-and-fire (or leaky integrator) model does not exhibit such non-uniqueness. Thorough analysis of this latter model with J. P. Keener and F. C. Hoppensteadt (University of Utah) further distinguishes parameter regimes where phase-locking behavior only is expected from those where the response may be either phase-locked or ergodic (aperiodic). A manuscript which also includes numerical and analytical calculation of phase-locking patterns as a function of stimulus frequency and modulation intensity has been submitted to SIAM J. of Applied Mathematics.

Dispersive aspects of impulse propagation for the non-space-clamped HH cable equations have been investigated numerically with R. Miller, (manuscript in preparation). Calculations of periodic traveling wave solutions show the decline in propagation speed with increasing frequency in an upper frequency range. At higher temperatures, e.g. 18.5°C, the speed versus frequency relation for a lower freqency range exhibits a local maximum at which the speed exceeds that of a solitary impulse. Numerical solutions to the full cable equations, without the assumption of steady propagation, predict how the speeds of successive pulses in response to repetitive stimulation can vary during propagation along a uniform fiber. A pulse which sees an immediately preceding short interspike interval may be initially slower and then accelerate as it falls behind its predecessor while certain longer interspike intervals may lead to pulses with supernormal speeds. Such variations in speed and interspike interval during propagation stand in contrast to the classical view of propagation of identical pulses with identical speeds.

Continued collaboration with D. Alkon (NINCDS, LB, Woods Hole), W. Rall and R. Miller has led to formulation, analysis and numerical investigation of a simple two compartment model (soma-dendritic arbor) to simulate subthreshold response to synaptic input of Hermissenda photoreceptor cells. Of particular interest is the effect of increased somatic resistance on EPSP amplitudes and their attenuation. Our calculations demonstrate explicitly augmentation, and its dependence on synaptic conductance time course, of an individual EPSP and enhanced temporal summation for an EPSP train when soma resistance is increased. (Dr. J. Rinzel).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 13,000-06 MRB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mathematics of kinetics and reaction-transport systems.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John Z. Hearon Chief, MRB MRB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.6	PROFESSIONAL: 1.0	OTHER: .6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project continues to focus upon applications of linear algebra, matrix and vector space methods to problems in biology. Applications are heavily involved in compartmental analysis; linear control theory, and generalized kinetics. No small part of the project is devoted to meeting purely mathematical problems which arise on their proper abstract grounds.

PHS-6040
(Rev. 10-76)

1. Pole Placement Theorem.

There is a growing (and very practical) interest in control theory in biological system theory. Further the concepts of controllability and observability are playing a great role in system identification and parameter estimation from observations.

An important control problem is that of output-feed-back control. Let the state vector x obey

$$\dot{x} = Ax + bu$$

where A is the system matrix, b the control vector and u the (scalar) controller. Let the output y be a linear scalar function of the state $y = c^* x$. Then the choice of $u = y$ gives

$$\dot{x} = (A + bc^*)x$$

as the equation for the state vector. Now there is a famous theorem which states that if the original system is state-controllable, then there exists a c such that the eigenvalues of $A + bc^*$ can be made any arbitrary set of numbers. We have discovered that while the theorem is true, no legitimate proof appears to exist in the literature. The most recent claim of a coordinate-free proof appeared in 1977 (Lin. Algebra and Appl. 18, 171). We have furnished a correct coordinate-free proof based in part on existing theorems in the mathematical literature and in part on our previous work (J. Res. Nat. Bur. Stds. 82, 129, 1977) on controllability. The theorem is stated as a general theorem on the spectrum of a rank-one perturbation of a nonderogatory matrix. It contains the control-theoretic version as a special case.

2. Residence time, transit times and all that.

We have previously (Math. Biosci. 15, 69, 1972) derived a general expression for the mean residence time for a compartmental system under arbitrary initial conditions. We have now extended this to arbitrary inputs. In any case the mean residence time is the proper average of the mean times for those particles which entered the i^{th} compartment and exited from the j^{th}, where the average is over all pairs i, j. In the general derivation we eschewed this messy detail. It becomes important 1) for the use of the washout curve in estimating residence times 2) for the use of steady-state output curves for the same purpose and 3) for the analysis of transient times in presteady state enzyme systems as reported last year, to be able to state the kind of averaging which occurs. We therefore have established the relation between the stationary input, initial condition experiment, and washout experiment in terms of what the relevant weights are for the averaging process.

Publications:

Hearon, J. Z.: On the completely symmetric compartmental system. Bull. Math. Biol., in press.

Hearon, J. Z.: A monotonicity theorem for compartmental systems. Math. Biosci., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 13,001-06 MRB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mathematical formulations and analysis relevant to experimental neurophysiology.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Wilfrid Rall	Senior Research Physicist	MRB NIAMDD
OTHER:	John Rinzel	Research Mathematician	MRB NIAMDD
	Robert Miller	Staff Fellow	MRB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.7	.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

For several years, this project has been concerned with constructing, developing and testing a group of interrelated mathematical models, relevant to experimental neurophysiology and neuroanatomy. Together these models provide a theory that can account for various sequences of events in the soma and dendritic branches of a single neuron, and for field potentials generated by certain cortical populations of neurons. Computational experiments performed with these models provide theoretical predictions that have been compared with experimental results obtained by colleagues with motoneurons of cat spinal cord, and with the mitral cell and granule cell populations of rabbit olfactory bulb. Resulting interpretations contribute to understanding of dendritic synaptic input and of dendro-dendritic synaptic interactions. These results have appeared in the Biophysical Journal and in the Journal of Neurophysiology. A comprehensive chapter, entitled "Core conductor theory and cable properties of neurons" (in The Handbook of Physiology: The Nervous System, Vol. I, Cellular Biology of Neurons, American Physiological Society 1977) summarizes some of these results.

PHS-6040
(Rev. 10-76)

1. Neural modeling that was originally directed at understanding certain experimental observations in photoreceptors of a nudibranch mollusc, has led to theoretical results and insights which have application to other nervous systems and to observations related to conditioning phenomena not only in insects and mollusks, but also in the mammalian central nervous system. Computations were aimed at distinguishing between different aspects of the long lasting depolarization (LLD) observed by Alkon and Grossman following light input to these molluscan photoreceptors. Their experiments showed that this LLD involves not only significant membrane depolarization of the photoreceptor soma, but also a very significant increase of soma membrane resistance. Our modeling enabled us to separate the effects of the resistance increase from the effects of the depolarization. We could demonstrate (both analytically and computationally) some important consequences of the membrane resistance increase. Briefly, the increase of soma membrane resistance not only increases the input resistance of the neuron measured either at the soma or in the dendrites, it also significantly increases the slowest membrane time constant, τ_0, of the model system. Although the increased input resistance did result in an expected increase of synaptic potential amplitudes when the synaptic activation had a slow time course, this effect was negligible for brief synaptic potentials. However, we predicted and then demonstrated computationally that the increase of τ_0 is responsible for a prolonged decay time and for a significant increase in the effectiveness of temporal summation of brief synaptic potentials (in both regular and stochastic temporal sequences). Now that this contribution to increased depolarization (by temporal summation of EPSP) has been demonstrated and understood theoretically, its contribution to the complicated associative behavioral changes observed by Alkon and colleagues can be explored by future modeling. Also, this effect seems likely to be relevant to cases of increased resistance observed following conditioning of neurons in the locust (Hoyle and colleagues at the Univ. of Oregon) and in the cerebral cortex of cat (Woody and colleagues at UCLA). [W. Rall collaborating with Drs. J. Rinzel and R. Miller, and with Dr. D. L. Alkon of NINCDS].

2. Detailed illustrative computations have been carried out and presented to biologist audiences to show them how the mathematical treatment of dendritic branching (Rall, 1959) can be applied to the wealth of new data now being obtained by many investigators. The extensive branching of individual neurons from many electrophysiological preparations can be revealed and measured with the help of Lucifer yellow, horseradish peroxidase, or cobalt with intensification by silver. Functional interpretation of such branching depends upon such biophysical computations. Such computations and interpretations were presented to a symposium on "Neurons without Impulses" for a particular nonspiking neuron whose very extensive branching was displayed by Burrows and Siegler (Cambridge, UK) using the cobalt technique with silver intensification. It was shown that calculations using trial values of 3000 ohm cm^2 for membrane resistivity and 83 ohm cm for core resistivity yielded computed input resistance values between 3.7 and 7.5 megohms (for the major processes) in good agreement with experiment. Calculations of voltage attenuation and functional distances between different dendritic arbors of the same neuron were presented and discussed, with regard to a possible role in changing the coupling between different neuronal activities that include nonspiking interneurons.

3. Biophysical analysis of dendritic spine stem resistance as an important parameter for modifications of synaptic potency. There has been continuing interest expressed by physiologists, anatomists and psychologists, both in workshops and in personal communications. This has led to calculations and discussions which serve to explore possibilities for future collaborations.

Publications:

Rall, W.: Dendritic spines and synaptic potency. In Porter, R. (Ed.): Studies in Neurophysiology. Great Britain, Cambridge Univ. Press, 1978, pp. 203-209.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 13,002-07 MRB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mathematical description of the substrates transport in the capillary-tissue structures.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jose M. Gonzalez-Fernandez	Research Mathematician	MRB NIAMDD
OTHER:	Susie E. Atta	Mathematician	MRB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	2.0	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To develop mathematical models of the blood flow and transcapillary exchanges in capillary networks. An effort is being made to incorporate in the models the histological structure of capillary networks as well as different flow patterns from available experimental information. In this model the extraction of substrates with different chemical kinetics at the tissue site will be described. It is expected that this could be used in experimental situations where the extraction of different substrates are measured simultaneously, thus helping to infer the flow pattern features of the microcirculation. In particular a model of the diffusion-consumption of oxygen in striated muscle containing myoglobin (facilitated diffusion) is being developed and pertinent numerical results examined.

PHS-6040
(Rev. 10-76)

Project Number: Z01 AM 13,002-07 MRB

A numerical method to solve for the facilitated transport of oxygen in the presence of hemoglobin or myoglobin was reported previously. With this method an extensive analysis of the available experimental information has been carried out. From the results obtained one can conclude that the observed facilitation can be accounted for by the simultaneous diffusion of oxygen, the protein and the oxygen-protein compound plus their chemical interaction. A paper containing these results has been submitted for publication. In the striated muscles there exist membranes, the sarcoplasmic reticulum and the muscle fiber membrane, that may impede the diffusion of the protein carrier. A theoretical study was undertaken to analyze the facilitated transport in the presence of membranes on the diffusion path. By using the spatial parameters and the chemical kinetics appropriate to the striated muscle a number of computations were carried out. The results indicate that the presence of those membranes may not interfere significantly with the facilitated transport in vivo. A paper with these results has been submitted for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 13,004-05 MRB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Mathematical description of cellular neuroelectric signal transmission

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John Rinzel	Research Mathematician	MRB NIAMDD
OTHER:	Wilfrid Rall	Senior Research Physicist	MRB NIAMDD
	Robert Miller	Staff Fellow	MRB NIAMDD

COOPERATING UNITS (if any)
R. Guttman, Res. Biologist
S. Lewis, Asst. Res. Biologist
Marine Biology Lab., Woods Hole, MA

F. C. Hoppensteadt, Prof./Mathematics
J. P. Keener, Assoc. Prof./Math.
Univ. of Utah
Salt Lake City, UT

LAB/BRANCH
Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.7	.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project continues to focus on the formulation, analysis, and biophysical interpretation of mathematical models which describe various aspects of neuroelectric signaling for individual neurons. Among the topics of current interest are:

(i) integration of synaptic input delivered to the soma and dendritic branches of a neuron;

(ii) steady propagation of action potentials along axons;

(iii) stimulus-response and threshold properties for repetitive firing of action potentials;

Mathematical models of these phenomena involve systems of linear and nonlinear ordinary differential equations and parabolic partial differential equations. Solutions and their mathematical stability are determined by analytical and numerical methods. One aspect in the approach of this project is to expose the qualitative mathematical structure for classes of models by exploiting simple, yet reasonable, equations.

PHS-6040
(Rev. 10-76)

1. (With Robert Miller) Our previous calculations for the space-clamped Hodgkin-Huxley (HH) equations provided a complete description of the primary branch family of periodic solutions as parameterized by the value for steady applied current I. This branch has a limb of stable solutions which joins a limb of unstable solutions at $I = I_v$ and describes the overlap range $I_v < I < I_1$ where there are two stable states, one oscillatory (repetitive firing) and one steady (maintained depolarization). Our further investigation has revealed, for the temperature 6.3°C, additional branches of periodic solutions which bifurcate off the unstable limb of the primary branch. These solutions appear as period-doubled (subharmonic) solutions and are unstable. There appears to be a cascade of such period-doubling bifurcations although this question cannot be resolved fully by numerical calculation. These results along with previous calculations and our specially developed numerical method to compute limit cycles directly are described in a manuscript soon to be submitted for publication. We expect the numerical method to be useful, and the detailed bifurcation diagrams to serve as illustrative examples, for nonlinear oscillation problems in a wide variety of applications.

(With R. Guttman and S. Lewis) The existence of an overlap range, as described above, where two distinct stable states are possible implies that hysteresis behavior would result in response to a slow current sweep upward and then back downward through the overlap range. Moreover for fixed I, $I_v < I < I_1$, appropriate brief finite amplitude shocks would cause the membrane to switch from one state to the other. These observations motivated an experimental-theoretical collaboration with R. Guttman and S. Lewis (MBL, Woods Hole). The experiments, carried out for squid axon bathed in low calcium, showed that repetitive firing in response to a just suprathreshold step, can be annihilated by a brief depolarizing or hyperpolarizing pulse of the proper magnitude applied at the proper phase. Moreover, hysteresis was demonstrated directly by using a slow up and then down current ramp. Physiological implications include stabilization against noise for near threshold behavior and a possible mechanism to start or stop a biological pacemaker with a brief synaptic input. These results were presented at the Biophysical Society Annual Meeting (February, 1979) and at the Gordon Research Conference on Theoretical Biology and Biomathematics (June, 1979); a manuscript has been submitted to J. Physiology.

2. (With J. P. Keener and F. C. Hoppensteadt) In projects of paragraph 1, periodic response to steady current was studied. Here a sinusoidally varying stimulus is considered. For certain stimulus parameter values, the HH equations and the FitzHugh-Nagumo equation may exhibit more than one stable phase-locking response. In contrast, the integrate-and-fire (or leaky integrator) model does not exhibit such nonuniqueness. Because this latter model involves only one dependent variable and is much simpler to solve we can provide a thorough analysis of its firing phase characteristics; we apply theorems on properties of mappings of the circle onto itself. We distinguish parameter regimes where phase-locking behavior only is expected from those where the response may be either phase-locked or ergodic (aperiodic). Parameter regions for 1:n phase-locking are characterized analytically. These results along with numerical examples of phase-locking patterns are described in a manuscript submitted to SIAM J. of Applied Mathematics, Part C, Biological Applications.

3. (With R. Miller) Dispersive aspects of impulse propagation along nerve fibers is of continued interest in this project. Our calculations of the dispersion relation for periodic traveling wave solutions to the HH cable equations show the decline in propagation speed and spike amplitude with increasing frequency in an upper frequency range. Moreover, at higher temperatures, e.g. 18.5°C, the speed versus frequency relation for a lower frequency range exhibits an obvious local maximum at which the speed exceeds that of a solitary impulse. This maximum is apparently analogous to the supernormal speed observed in two-pulse experiments in some preparations: a test pulse which follows a conditioning pulse by a certain interval is seen to have a shorter conduction time. This phenomenon which has been attributed to the spike after-depolarization can be clearly understood in terms of the computed ionic conductance trajectories during the return to rest. Additional numerical solutions to the full cable equations, without the assumption of steady propagation, show how the speeds of successive pulses in response to repetitive stimulation can vary, because of dispersion, during propagation along a uniform fiber. A pulse which sees an immediately preceding short interspike interval may be initially slower and then accelerate as it falls behind its predecessor while certain longer intervals may lead to pulses with supernormal speeds. These results may be considered predictive for squid axon. More generally, such variations in speed and interspike interval during propagation stand in contrast to a classical view of propagation of identical pulses with identical speeds. Hypotheses of temporal coding should not be made without assessment of the effects of dispersion.

4. (With D. Alkon, R. Miller and W. Rall) Dr. Alkon has observed a period of increased soma membrane resistance along with long lasting depolarization following a light flash to photoreceptors in Hermissenda. He has conjectured that such increases lead to augmentation of EPSP's for synaptic input at dendritic arbor locations. We have formulated a two compartment model (soma-dendritic arbor) with passive membrane characteristics; ranges for parameter values were estimated from Alkon's measurements. The response for a transient synaptic conductance change was studied numerically as a function of model parameters. Increased somatic resistance leads to augmentation of an individual EPSP (the effect is greater for slower transients than faster ones) and enhanced temporal summation for an EPSP train.

Publications:

Rinzel, J.: Integration and propagation of neuroelectric signals. In Levin, S. A. (Ed.): MAA Studies in Mathematical Biology. Washington, D. C., Mathematical Association of America, 1978, pp. 1-66.

Rinzel, J.: On repetitive activity in nerve. Federation Proc. 37: 2793-2802, 1978.

Annual Report of the Arthritis and Rheumatism Branch

National Institute of Arthritis, Metabolism and Digestive Diseases

The Section on Human Biochemical Genetics was separated from the Branch and became the nucleus of a new Genetics and Biochemistry Branch, Dr. Elizabeth F. Neufeld, Chief.

A pilot study of lymphocyte depletion in four patients with rheumatoid arthritis produced unexpectedly excellent results with major and sustained recession of disease activity at the cost of virtually no toxicity (ZO1 AM 41035). The depletion was carried out with a continuous flow cell separator using an arteriovenous shunt for venous access. Four hour "treatments" were carried out three times weekly for 5 weeks. In addition to the selective removal of lymphocytes and monocytes, small amounts of plasma were removed with each run. The next step was to establish that the effect was due to mononuclear cell depletion and to this end a study has been started comparing, under double-blind circumstances, mononuclear cell and plasma removal to plasma only removal (ZO1 AM 41044). The work has proceeded more rapidly because of the installation on the Clinical Center 9 East Nursing Unit of the necessary equipment for a "pheresis room."

Studies of the control of rheumatoid arthritis with intermittent intravenous cyclophosphamide are also in progress (ZO1 AM 41042). The modality was chosen because of the known effectiveness of oral cyclophosphamide and the fact that the intermittent treatment plan permits better protection of the urinary bladder mucosa by urine dilution during the limited period when the irritating metabolites are most concentrated in the urine.

The major clinical studies of the Branch focus on systemic lupus erythematosus (SLE). The extended follow-up required by these studies has been facilitated by a developing system for clinical machine data handling (ZO1 AM 41029). The CAP (ZO1 AM 41000) and PACIV (ZO1 AM 41040) studies, directed toward the control of SLE nephritis, continue active with new patients who qualify being admitted to the latter. The work has shown that, in the chronic process involved, the simple addition of cyclophosphamide or azathioprine to baseline corticosteroid therapy is not advantageous to the patient. On the other hand, intermittent intravenous cyclophosphamide and a combined program of triple drug therapy (cyclophosphamide, azathioprine, and prednisone) appears to be producing improved results. Intensive light and electron microscopic study of renal biopsy specimens has permitted more precise classification of the nephritis and estimates of its prognosis (ZO1 AM 41038). Elegant studies of the newly available pneumococcal vaccine and its effect on patients with SLE under various forms of treatment are in progress (ZO1 AM 41043). Prostaglandin synthetase inhibiting drugs have been found sharply to reduce renal creatinine clearance most prominently in patients whose compromized renal blood flow is partially maintained by prostaglandins (ZO1 AM 41028).

Several recent reports have raised a possible relationship between SLE and a spontaneous disease of dogs called canine SLE, which, among other features, is characterized by serum antinuclear antibody (ANA). With the help of a number of major veterinary pathological laboratories, a series of ANA positive dogs were identified and a matched group of ANA negative dogs were selected. Interviews and blood sampling of all persons in the households from which these animals came were carried out. No differences in any measure, either clinical or with sensitive laboratory tests, were detected between the two types of households (Z01 AM 41036).

Studies of lymphocytes of a large number of our patients with SLE have established rather striking relationships between the disease and certain genetic markers of the major histocompatibility complex (Z01 AM 41037). One B-lymphocyte alloantigen, for example, was found in 72% of the patients and only 14% of the controls, a relative risk of 15.4. Studies of families with multiple cases of SLE are in progress and strongly support the concept that coinheritance of several such antigens influence susceptibility to the disease.

The manifestations of SLE are thought to be mediated by immune complexes; in the normal individual complexes are rapidly removed by the reticulo-endothelial system but studies in collaboration with NIAID scientists show very much delayed clearance in patients with active SLE (Z01 AM 41030). In addition to circulating complexes, the abnormal relationships between T and B lymphocytes and, among the T-lymphocytes, between T-helper and T-suppressor cells which exist in SLE have been under study (Z01 AM 41023). For example, in contrast to normals, SLE T-cells activated by Concanavalin A do not generate suppressor signals. The studies are made difficult to perform and analyze by the fact that many T-cell functional abnormalities appear to be induced by anti-T cell antibodies in the plasma rather than being determined by intrinsic cellular abnormalities.

In addition and complementary to work with the disease in patients, model immune complex disposition (Z01 AM 41018) and the spontaneous murine lupus of New Zealand mice (Z01 AM 41020, 41021, 41022) are under study. The latter studies have emphasized the genetic basis for autoimmunity which is prominent and have also demonstrated the major role played by sex hormones in the expression of autoimmunity. The model immune complexes can be prepared in various sizes and their study has shown that they are removed from the circulation at a rate inversely related to their size.

Studies of the IgE receptor system, as a model for the study of mechanisms of antigen-induced antibody-mediated cellular reactions, has made progress particularly with the recognition that, while IgE dimers were a "sufficient" trigger, higher aggregates in fact induced greater release by the cells (Z01 41025). The membrane bearing the receptor and the receptor itself are being analyzed, the former in functional assays of inverted plasma membrane vesicles and the latter by appropriate cleavage followed by peptide and sequence analysis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41000-10 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Controlled study of cyclophosphamide and azathioprine in systemic lupus erythematosus with nephritis (CAP)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.L. Decker	Chief, Arthritis and Rheumatism Br.	ARB NIAMDD
	A.D. Steinberg	Senior Investigator	ARB NIAMDD
OTHER:	P.H. Plotz	Senior Investigator	ARB NIAMDD
	J.H. Klippel	Senior Investigator	ARB NIAMDD
	J.E. Balow	Senior Investigator	ARB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	1/2	1/4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with diffuse glomerulonephritis and systemic lupus erythematosus satisfying criteria for active renal disease are offered a therapeutic study. The patients are randomized to different treatment programs which include the following drugs: corticosteroids, azathioprine and cyclophosphamide.

Follow-up visits occur every six months and the patients are followed until death or dialysis. Clinical and immunologic assessment are performed. The risks and benefits of each treatment will be evaluated and compared.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Two ten week studies by our Branch suggested that cyclophosphamide was effective in the treatment of lupus nephritis and that azathioprine was less effective. A long-term study of these patients is being carried out to determine whether or not one therapeutic program is superior over the course of several years.

Methods Employed:

Patients meeting predefined criteria for diagnosis of SLE and activity of nephritis have been admitted to the trial. In a baseline control period, the patients were kept on the anti-inflammatory regimen (usually prednisone and aspirin) needed to control extra-renal manifestations. These have been continued with as little change as possible.

A large number of serological, chemical and urine values are serially obtained and the patients will be followed for life. Immunoglobulin levels, anti-DNA antibodies, and serum complement are among those measures. We will determine time to death or renal dialysis in each treatment program and assess the toxicity as well as efficacy of each program.

Major Findings:

The first patient was admitted to this trial in April 1970 and the 38th and last in October 1972.

After courses ranging up to 7 years duration, we were unable to detect an advantage conferred by cyclophosphamide or azathioprine to corticosteroids alone. Furthermore, we were increasingly impressed with the untoward effects of cyclophosphamide, particularly hemorrhagic cystitis and carcinoma of the bladder. In contrast, azathioprine appeared quite safe.

Accordingly in March 1979 a plan was developed for more intensive observation of the patients over a 9 month period extending from before to well after cytotoxic drug withdrawal; drug withdrawal is now in progress.

Significance to Bio-Medical Research and the Program of the Institute:

This work has already had wide medical impact. The change should allow us to draw conclusions in reference to the hazards of drug withdrawal if any.

Publications:

Information gained in this study has been reported in references cited with Z01 AM 41040-07.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41001-08 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Therapy of SLE: A controlled trial comparing high dose intravenous cyclophosphamide with placebo.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Plotz	Senior Investigator	ARB NIAMDD
OTHER:	J.L. Decker	Chief, Arthritis and Rheumatism Br.	ARB NIAMDD
	A.D. Steinberg	Senior Investigator	ARB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1/4	PROFESSIONAL: 0	OTHER: 1/4

CHECK APPROPRIATE BOX(ES)
X
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

At present no satisfactory therapy exists for severe manifestations of SLE except for renal disease. Even renal disease does not respond well to conventional therapy when the creatinine clearance is markedly reduced. In this study we will test the efficacy of high dose intravenous therapy with cyclophosphamide for the control of severe SLE unresponsive to a trial of high doses of prednisone (up to 2 mg per kg for three weeks).

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

At present no satisfactory therapy exists for severe manifestations of SLE except for renal disease. Even renal disease does not respond well to conventional therapy when the creatinine clearance is markedly reduced. In this study we will test the efficacy of high dose intravenous therapy with cyclophosphamide for the control of severe SLE unresponsive to a trial of high dose of prednisone (up to 2 mg per kg for 3 weeks).

Methods Employed:

Patients with 1) severe renal disease or rapid deterioration or renal function, 2) sensitivity to azathioprine precluding the CAP protocol, 3) severe extra-renal lupus (seizures, hemorrhage, psychosis, hemolytic anemia, myocarditis, or pericarditis uncontrolled by high-dose prednisone) will be randomly assigned to receive intravenous cyclophosphamide or placebo for 3 initial injections over 6 weeks and then at 3 month intervals. Renal and non-renal patients will be separately randomized. The study will be double-blind: neither the physicians nor patients will know which therapy is being administered. The end point of the study will be death or the need for renal dialysis except that patients well for 1 year may have therapy stopped. The potential hazards and benefits will be explained in full to all patients or their families.

Major Findings:

No patients were entered during the past year.

Significance to Bio-Medical Research and the Program of the Institute:

The therapy of SLE has been under study here for several years. The present study is an attempt to extend the therapy for patients unresponsive to conventional therapy.

Proposed Course:

We plan to enter 20 to 30 patients into the study over the next 3-5 years.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41018-05 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Production of covalently cross-linked immune complexes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Plotz	Senior Investigator	ARB NIAMDD
OTHER:	D. Finbloom	Clinical Associate	ARB NIAMDD
	D. Magilavy	Clinical Associate	ARB NIAMDD
	A. Rifai	Staff Fellow	ARB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 1/2	2	1/2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies have been site-specifically covalently cross-linked with a newly synthesized class of compounds which act as bis-affinity-labeling hapten. These oligomers of IgG are used as model immune complexes. They are separated by size and the biological properties of the sized oligomers are studied. Properties presently under study include complement fixation and Clq binding and biological survival.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To study the role of the configuration of immune complexes in determining their pathogenetic properties.

Methods Employed:

Anti-hapten antibodies are site-specifically cross-linked and the resulting oligomers are separated by size. Biological survival and complement fixation are studied in the standard fashion.

Major Findings:

Oligomers are removed from circulation with a rate inversely proportional to their size. Rate of removal is not apparently related to complement. Complement is not fixed in solution by small oligomers, but is fixed when oligomers are bound to the surface of red blood cells.

Significance to Bio-Medical Research and the Program of the Institute:

This work may allow studies of the properties of stable immune complexes of various sizes relevant to their role in the pathogenesis of connective tissue disease.

Proposed Course:

During the coming year, Dr. Finbloom will continue studies on the capacity of the RES to handle immune complexes; Dr. Finbloom and Dr. Magilavy will study the binding of model immune complexes to macrophages from organs with different capacities to take up complexes in vivo. Studies on complement fixation will continue, using covalent oligomers formed with larger, flexible, affinity-labeling haptens. Dr. Rifai will study the disposal of complexes prepared with antigens of different overall electrical charge.

Publications:

1. Plotz, P.H., Kimberly, R.P., Guyer, R.L., and Segal, D.M.: Stable model immune complexes produced by bivalent affinity-labeling haptens: In vivo survival. Molecular Immunol. (in press), 1979.

2. Segal, D.M., Guyer, R.L., and Plotz, P.H.: Complement fixation by model immune complexes free in solution and bound onto cell surfaces. Biochemistry 18: 1830-1835, 1979.

3. Jones, J.F., Plotz, P.H., and Segal, D.M.: Complement and cell-mediated lysis of haptenated erythrocytes sensitized with oligomers of rabbit IgG antibody. Molecular Immunol. (in press) 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41020-12 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Pathogenesis of autoimmunity in New Zealand mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.D. Steinberg	Senior Investigator	ARB/NIAMDD
OTHER:	D. Huston	Clinical Associate	ARB/NIAMDD
	J. Taurog	Clinical Associate	ARB/NIAMDD
	P. Smathers	Biologist	ARB/NIAMDD
	J. Reeves	Chemist	ARB/NIAMDD
	E. Raveche	Chemist	LEP NIAMDD
	K. Melez	Guest Worker	PMB NIAMDD

COOPERATING UNITS (if any)
Laboratory of Experimental Pathology, NIAMDD

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3 1/2	3 1/4	1/4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The pathogenesis of autoimmunity in New Zealand mice is still uncertain; however, advances are being made. The disease has a genetic and immune basis; however, expression of severity can be modified by viral and other environmental factors as well as sex hormones. The immunologic factors are complex. These include excessive B-cell activation and impaired T-cell suppressive activity. The loss of T-cell activity is related to a deficiency of suppressor cell precursors. Antibodies to T-cells produce an accelerated loss of suppressor cells and their precursors. Excessive B-cell activation can be modified by introducing the X chromosome of a CBA/N mouse into the NZB autoimmune background. The CBA/N X chromosome which codes for defects in B-cell maturation causes a reduction in autoimmunity in F_1 mice, and in preliminary experiments in congenic mice. Male sex hormones present in the first few weeks of life markedly retard anti-DNA production later in life.

PHS-6040
(Rev. 10-76)

Project Description:

Objective:

New Zealand mice spontaneously develop an autoimmune disease resembling human systemic lupus erythematosus. Early in life they are immunologically hyperactive, become relatively resistant to tolerance induction, and gradually develop antinuclear and other autoantibodies followed by clinical disease. Both humoral and cellular immune defects have been reported and are under active investigation. In addition, the bases for the differences in disease expression in New Zealand mice are being explore.

Methods Employed:

1. Assay for antibodies to RNA and DNA using ^{14}C-labeled nucleic acids as ligands and the ammonium sulfate precipitation assay. Assay of antibody forming cells to sheep erythrocytes and pneumococcal polysaccharide by local hemolysis in gel.

2. Immunization of animals with a variety of antigens with and without protein carriers or adjuvants.

3. Transfer of spleen, bone marrow and thymus cells into recipients to evaluate the functional properties of the different cell types.

4. Use of standard skin grafting using allogenic skin to evaluate cellular immune functions in New Zealand mice, and transfer of serum and cell population between young and old New Zealand mice.

5. Study of graft versus host disease inducing properties of NZB/W lymphoid cells using the Simonsen spleen weight assay and newborn C3H/He or SWISS mice as recipients, or adult athymic (nude) mice.

6. Separation of lympoid cells functionally (corticosteroid, anti-T cell sera, anti-kappa) and physically (sedimentation).

7. Assay of anti-T cell antibodies by release of ^{51}Cr from thymocytes.

8. Evaluation of subpopulation of T-cells using the fluorescence activated cell sorter.

9. Castration of male and female NZB/W mice at various ages and treatment with sex hormones by either injection or implantation of sialastic tubes containing crystaline hormones.

10. Immunization of mice with H-2 incompatible cells and studying in vitro regulation of T-cell function: proliferation in response to, and killing of, the H-2 incompatible cells.

Major Findings:

1. NZB hybrids demonstrate a marked sex difference in spontaneous autoantibody production: Females produce autoantibodies earlier and at higher titers than males. Castration, but not vasectomy, of NZB X DBA/2 males increased NTA antibody production to the levels seen in females. Treatment with testosterone, but not estradiol, retards the accelerated production of NTA in females and castrated males. Castration of NZB/NZW males accelerates their disease which is prevented by testosterone but not estradiol. Castration of females by itself has little effect; however, castration plus testosterone retards disease.

2. Treatment of NZB/NZW females with testosterone starting at 2-3 weeks of ages leads to inhibition of anti-DNA production. Treatment at 15 weeks of age leads to no change in anti-DNA, but prolonged survival.

3. Susceptibility to spontaneous anti-DNA is inherited as a single dominant trait. This is not linked to production of anti-T-cell antibodies which is controlled by a codominant trait.

4. Immunization of NZB hybrids with DNA demonstrated that high responder status is inherited as a dominant autosomal trait which is modified by sex hormones: androgrens suppress and estrogens accelerate. CBA/N mothered male hybrids with an x-linked defect in responsiveness to rI•rC failed to be immunized even if castrated and treated with estrogens.

5. The cytotoxic lymphocyte responses of NZB mice were suppressed by allosensitized spleen cells but not by suppressor factors elaborated by such cells. This defect was found to be a recessive trait.

6. Injection of 6 week old NZB/NZW females with parental NZB or NZW spleen cells caused acceleration of the production of non-pathogenic autoantibodies, retardation of pathogenic autoantibodies, and reduced proteinuria plus prolonged survival.

7. NZB mice and their F_1 hybrids produce excessive polyclonal IgM and autoantibodies of both IgM and IgG classes. CBA/N mice and CBA/N mothered F_1 males fail to make antibody to many T-independent antigens and have low levels of serum IgM; further, these mice lack a population of splenic B-cells characterized by a low-to-intermedate density of surface IgM. The CBA/N X-linked defect of T-independent immune response is completely expressed in CBA/N X NZB mice. In marked contrast to NZB mice and to NZB F_1 hybrids bearing at least one normal X-chromosome, the CBA/N X NZB males failed to respond to two T-independent antigens, had small numbers of splenic IgM producing cells, barely detectible splenic IgM production, and splenic B-cell surface immunoglobulin patterns resembling those of CBA/N

mice. These data suggest that the NZB B-cell abnormality resulting in excessive IgM production occurs almost exclusively in that population of B-cells affected by the CBA/N X-chromosome linked defect. Preliminary studies suggest that the CBA/N X-chromosome retards the spontaneous development of anti-erythrocyte autoantibodies in CBA/N X NZB males.

Proposed Course:

The role of genetic factors will be further investigated. This will include study of congenic mice on a NZB background but with B-cell hypoactivity as well as recombinant inbred lines. The availability of athymic nude mice will allow us to study lack of thymic function in different strains with and without superinfection with different viruses or immunization with nucleic acid antigens. We hope thereby to define the relative contributions of genetic and different immunologic factors in the development of autoimmunity. These studies will provide evidence regarding the relative importance of B-cell defects and T-cell defects in the development of autoimmunity. This function will be further addressed in transfer studies. The role of anti-idiotypic antibodies in control of autoantibody production will be investigated.

Significance to Bio-Medical Research and the Program of the Institute:

1. The role of sex hormones in modulating autoantibody production provides a means by which disease modulation may be better understood, investigated and perhaps altered.

2. The genetic basis for autoimmunity is becoming much better established. The studies of B-cell defective mice point to potential genetic manipulations.

3. Study of immune regulation provides a greater understanding of the details of disordered immune regulation in New Zealand mice and points to therapeutic manipulations in the future.

Publications:

1. Gershwin, M.E., and Steinberg, A.D.: Graft versus host reactions induced by administration of parental cells: Effect on the autoimmune process of NZB/NZW F_1. Clin. Immunolo. Immunopathol. 10: 403-413, 1978.

2. Melez, K., Reeves, J.P., and Steinberg, A.D.: Modification of NZB/NZW disease by sex hormones. J. Immunopharmacol. 1: 27-42, 1978.

3. Melez, K., Reeves, J.P., and Steinberg, A.D.: Modification of murine lupus by sex hormones. Annales d'immunologie 129C: 707-714, 1978

4. Raveche, E.S., Tjio, J.H., and Steinberg, A.D.: Hyperdiploidy in NZB mice and their hybrids. Cytogenetics and Cell Genetics (in press), 1979.

5. Huston, D.B., Rich, R.R., Steinberg, A.D., and Truitt, G.A.: Regulation of cytotoxic lymphocyte responses in New Zealand mice by alloantigen-activated spleen cells. J. Immunol. (in press)., 1979.

6. Raveche, E.S., Tjio, J.H., and Steinberg, A.D.: Genetic studies in NZB mice. III. Induced antinucleic acid antibody production. J. Immunol. (in press), 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41021-11 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Antinuclear antibodies in spontaneous and drug-induced SLE and other diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.D. Steinberg	Senior Investigator	ARB NIAMDD
OTHER:	D. Eilat	Visiting Scientist	LCB NIAMDD
	E.S. Raveche	Chemist	LEP NIAMDD
	J.P. Reeves	Chemist	ARB NIAMDD
	K. Melez	Guest Worker	PMB NICHD

COOPERATING UNITS (if any)
Laboratory of Chemical Biology, NIAMDD
Laboratory of Experimental Pathology, NIAMDD

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 1/2	2 1/2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies to nucleic acids are important diagnostically and pathogenetically in systemic lupus. The production of these antibodies is in part controlled by the X-chromosome through regulation of maturation of B cells destined to make IgM antibodies to nucleic acids. Sex chromosomes indirectly influence the magnitude to auto-antibody production through sex hormones: male sex hormones suppress the production of anti-nucleic acid antibodies. Antibodies to transfer RNA are produced by patients with SLE; these cross-react with viral nucleic acids. Subpopulations of patients have specific auto-antibodies.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Study the characteristics of a variety of antinuclear antibodies in human and murine "autoimmune diseases." Determine genetic contributions to the immune response to nucleic acid and related antigens.

Methods Employed:

Farr ammonium sulfate precipitation assay using radiolabeled ligands. Standard precipitin reaction using labeled and unlabeled nucleic acids or nuclear extracts. Hemagglutination, complement fixation and immunodiffusion assays. Standard immunization procedures.

Major Findings:

1. Genetic studies of NZB x DBA F_1 and backcross mice indicate that a limited number (1 or 2) of genes play the major role in controlling production of anti-DNA.

2. Castration of male NZB x DBA or DBA x NZB mice led to accelerated anti-DNA production, both following immunization and spontaneously. This was suppressed by testosterone and further accelerated by estrodiol. Castration of 3 week old NZB/NZW males accelerates anti-DNA production; castration at 3 months does not. Testosterone treatment of 3 week NZB/NZW females retards anti-DNA production; treatment at 3 months does not.

3. Patients with SLE and NZB/NZW mice spontaneously produce antibodies reactive with native RNA species. An *in vitro* technique has been devised for precise measurement of such antibodies produced by cells in culture.

Significance to Bio-Medical Research and the Program of the Institute:

Male sex hormones appear to suppress auto-antibody production explaining the more severe disease and greater incidence in females. This effect occurs primarily very early in life; it suggests that hormones modulate a maturational event in the immune system which in turn regulates autoimmune potential. Genes for high and low responsiveness to DNA may be one of the genetic contributions to autoimmunity.

Proposed Course:

Further define the genetics of the antibody response to DNA and try to correlate with other immune abnormalities. Further study the characteristics of antinuclear antibodies. Continue to evaluate the role of sex hormones in autoimmunity. Compare antinuclear antibodies of patients with different autoimmune diseases. Further study subpopulations of patients with a high incidence of autoimmunity.

Publications:

1. Eilat, D., Reeves, J.P., Steinberg, A.D., and Schecter, A.N.: Precise measurement of antigen-specific IgG autoantibody production in vitro. J. Immunol. 121: 310-314, 1978.

2. Gershwin, M.E., and Steinberg, A.D.: Graft versus host reactions induced by administration of parental cells: Effect on the autoimmune process of NZB/NZW F_1 mice. Clin. Immunol. Immunopathol. 10: 403-413, 1978.

3. Melez, K., Reeves, J.P., and Steinberg, A.D.: Modification of NZB/NZW disease by sex hormones. J. Immunopharmacol. 1: 27-42, 1978.

4. Pincus, T., Steinberg, A.D., Blacklow, N.R., and Decker, J.L.: Reactions with cellular and virus antigen preparations in sera of patients with systemic lupus erythematosus. Arth. Rheum. 21: 873-879, 1978.

5. Melez, K., Reeves, J.P., and Steinberg, A.D.: Modification of murine lupus by sex hormones. Annales d'immunologie 192C: 707-714, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41022-08 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Therapeutic studies in New Zealand mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.D. Steinberg	Senior Investigator	ARB NIAMDD
OTHER:	N.L. Gerber	Chief, Rehabilitation Dept.	CC REHAB
	J.P. Reeves	Chemist	ARB NIAMDD
	K. Melez	Staff Fellow	PMB NICHD
	P. Smathers	Biologist	ARB NIAMDD

COOPERATING UNITS (if any)
Rehabilitation Department, Clinical Center

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Non-specific immunosuppressive therapy of man is associated with considerable morbidity. Some regimens may be superior to others. The New Zealand mice offer a convenient model for studying different drug regimens prior to study in patients. Soluble suppressor substances have prolonged life in NZB/NZW mice.

Treatment of NZB/NZW mice with Ribavirin, a broad spectrum antiviral drug, has been very effective. Renal deposits of immune complexes can be prevented. Such treatment is potentially applicable to humans with SLE.

Male sex hormones, testosterone and 5 alpha-dihydrotestosterone (but not Danazol) prolonged life in female NZB/NZW mice. Such treatment was effective even when started after clinical illness and did not require castration for its full effect.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

NZB/NZW F_1 mice spontaneously develop antibodies to nucleic acids and die of immune complex glomerulonephritis. NZB mice develop Coomb's positive hemolytic anemia, membranous nephrosis and excessive lymphoreticular proliferation. These mice are excellent models for comparing different immunosuppressive and anti-viral drug programs. In addition, as the immunologic basis for the disease becomes better understood, they offer a model for studying more specific immunotherapy.

Methods Employed:

1. Comparison of different immunosuppressive and anti-viral drug regimens in natural history of NZB/NZW mice by study of antibodies to DNA, proteinuria and survival.

2. Treatment with suppressor cells and/or suppressor substances derived from such cells in an attempt at restoring normal regulatory functions and improve the natural history of New Zealand mice.

3. Treatment of NZB/NZW and NZB mice with androgens.

Major Findings:

1. Ribavirin, a broad spectrum anti-viral agent, was found to be effective (a) prophylactically and (b) therapeutically in NZB/NZW mice. The favorable effects included: decreased proteinuria, decreased anti-DNA antibodies, and increased survival. No toxicity was observed. Little immunosuppression was seen. Ribavirin was at least as effective as cyclophosphamide.

2. Supernatants from BALB/c spleen cells stimulated with Concanavalin A were given to NZB/NZW mice starting at 18-19 weeks of age. Such mice had prolonged survival as compared with mice receiving inactive control supernatants. The 10,000 molecular weight fraction was at least as effective as the whole supernatant.

3. Androgens prolong life in female NZB/NZW mice when treatment is started before and when started after the onset of illness. Both testosterone and dihydrotestosterone were therapeutically effective in a dose-dependent manner. Danazol was of no benefit.

Significance to Bio-Medical Research and the Program of the Institute:

These studies are designed to provide background experience for clinical studies in which drugs may be given in a more effective manner to humans with SLE. Trials of the anti-viral drug Ribavirin will be considered in humans on the basis of these studies. Possible hormonal intervention will be considered in the future.

Continued study in mice may provide the background information necessary for immunological intervention in humans with SLE. Suppressor factors may be of value in humans in the future.

Proposed Course:

Further study of sex hormones in other SLE strains of mice, especially NZB and MRL, to determine whether or not such treatment is uniformly effective.

Publications:

1. Steinberg, A.D., and Decker, J.L.: Immunoregulatory Drugs. In McCarty, D.J. (Ed.): Arthritis and Allied Conditions. Philadelphia, Lea & Febiger, 1979, Chapter 25, pp. 375-390.

2. Melez, K., Reeves, J.P., and Steinberg, A.D.: Modification of NZB/NZW disease by sex hormones. J. Immunopharmacol. 1: 27-42, 1978.

3. Melez, K., Reeves, J.P., and Steinberg, A.D.: Modification of murine lupus by sex hormones. Annales d'immunologie 129C: 707-714, 1978.

4. Klassen, L.W., Williams, G.W., Reinertsen, J.L., Gerber, N.L., and Steinberg, A.D.: Ribavirin treatment in murine autoimmune disease. I. Therapeutic efficacy and effect on the immune response. Arth. Rheum. 22: 145-154, 1979.

5. Steinberg, A.D., and Steinberg, S.C.: Autoimmune disease and the clinical consequences of prolonged immunosuppression. In Proceedings of Symposium on Immunosuppression, held August, 1978, Food and Drug Administration (in press), 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41023-05 ARB

PERIOD COVERED
October 1, 1978 to September 31, 1979

TITLE OF PROJECT (80 characters or less)
Study of Immune Abnormalities in Patients with Systemic Lupus Erythematosus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.D.Steinberg	Senior Investigator	ARB NIAMDD
OTHER:	J. P. Reeves	Chemist	ARB NIAMDD
	D. Huston	Clinical Associate	ARB NIAMDD
	E.B. Merchant	Director	DBBP BB
	I. Green	Senior Investigator	LCI NIAID
	T. Sakane	Visiting Fellow	LCI NIAID
	R.M. Blaese	Senior Investigator	IB NCI
	J. Grayson	Clinical Associate	IB NCI

COOPERATING UNITS (if any)
Immunology Branch, NCI
Bureau of Biologics
Laboratory of Clinical Investigations, NIAID

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6 1/4	6	1/4

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Excessive B cell activity and loss of regulatory cells occurs in patients with active SLE; these abnormalities are less marked when the disease is quiescent. Nevertheless, excessive B cell proliferation occurs even in inactive patients.

Loss of a subpopulation of regulatory T cells in active SLE is caused by antibodies to these, spontaneously produced by the patients. Such antibodies prevent normal T cell control of abnormal B cell activity allowing perpetuation of autoantibody production. Some patients produce antibodies to a subset of T cells which regulates T cell but not B cell immunity. Such patients are less ill than those which produce antibodies capable of interfering with regulation of B cell function.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To apply the newer understanding of basic immunologic principles and observations in New Zealand mice (who develop a lupus-like disease) to the study of patients with SLE.

Methods Employed:

1. Study of families of patients with SLE or larger groups of patients with a high incidence of SLE. A battery of immunologic measurements would be made in such patients.

2. Study of lymphocytes and antibodies to lymphocytes from patients with SLE.

   a. Distribution on discontinuous gradients.

   b. Number and distribution of T, B and null cells and their responses to mitogens or allogeneic cells.

   c. Study of antibody producing plaque forming cells (PFC) in the peripheral blood of patients.

   d. Study of generation of suppressor cells by activating purified T cells with concanavalin A and adding them to a second culture. Study of other T-B interations with the autologous MLR.

   e. Relationship between anti-lymphocyte antibodies in patients with SLE and abnormalities of cell number and/or function.

   f. Study of anti-lymphocyte antibodies by use of the fluorescence activated cell sorter (FACS).

   g. Study of anti-lymphocyte antibodies by complement-mediated lysis.

Major Findings:

1. Anti-lymphocyte antibodies occur in association with increased activity of patients with SLE. These antibodies can reduce the number of peripheral T cells and interfere with their function, especially suppressor cell generation.

2. Antibodies to human lymphocytes found in sera from patients with SLE are readily studied on the FACS. IgM antibodies to T cells recognize the T cells of normals and patients with SLE. Such antibodies preferentially kill suppressor cells *in vitro* and probably preferentially inactivate them *in vivo*.

3. Purified T cell populations were activated with Concanavalin A and studied for ability to suppress B and T cell functions. SLE patient's T cells were defective in their ability to generate suppressor signals, but were able to respond to normal suppressor signals.

4. Patients with active SLE have increased quantities of antibody producing PFC to defined chemical haptens. Increases in IgG antibody producing cells especially characterized patients with active SLE.

5. Antibodies to T cells present in the plasma of patients with active systemic lupus erythematosus (SLE) plus complement are able to eliminate concanavalin A-induced suppressor function for the proliferative responses of T cells to allogeneic lymphocytes (MLR) and of B cells to pokeweed mitogen (PWM). Such antibodies were found to be effective in eliminating suppressor function only when T cells were treated prior to activation; there was no effect when treatment was performed after activation. These studies indicate that the antibodies preferentially interact with a T cell necessary for the generation of suppressor cells, rather than with mature, activated suppressor cells. Studies of individual SLE patients indicated that the same defects observed in SLE T cells were induced in normal T cells by plasma from that patient. Such observations suggest that many T cell defects associated with active SLE may not be intrinsic T cell abnormalities, but, rather, secondary effects of anti-T cell antibodies.

6. Studies of the T cell subpopulations responsible for suppression of the MLR and PWM responses indicated that only T-gamma cells acted as precursors of suppressor cells for the MLR, whereas both T-gamma and T-non-gamma cells could be activated to suppress the PWM responses. Consistent with this observation, SLE anti-T cell antibodies which preferentially killed T-gamma cells preferentially eliminated suppressor cells for the MLR. Finally, dose response curves showed that small numbers of both T-gamma and T-non-gamma cells increased the PWM response, suggesting that there are both T-gamma and T-non-gamma helper cells for the PWM response.

Significance to Bio-Medical Research and the Program of the Institute:

Populations for further study are being defined and techniques refined for continued study of humans with SLE. It is hoped that the rewarding observations in the animal model of SLE, New Zealand mice, can continue to be applied to the study of the human disease itself.

The studies to date suggest that patients with SLE have generalized B cell hyperactivity rather than selective B cell hyperactivity towards self-antigens. However, both genetic factors and environmental exposure determine which B cell clones will be expanded. Loss of suppressor and helper cells in human SLE is associated with (a) disease activity and (b) anti-T-cell

antibodies, suggesting that the lesion is not permanent and that appropriate specific prevention may be possible. The T cell dysfunction may largely be secondary to anti-T cell antibody production. This will direct primary therapeutic attention towards the excessive B cell activity and away from defective T regulatory function.

Proposed Course:

Continue the studies initiated.

Publications:

1. Sakane, T., Steinberg, A.D., and Green, I.: Studies of immune functions of patients with SLE. I. Failure of suppressor T cell activity related to impaired generation of, rather than response to, suppressor cells. Arthritis Rheum. 21:657-664, 1978.

2. Sakane, T., Steinberg, A.D., and Green, I.: Failure of autologous mixed lymphocyte reactions between T and non-T cells in patients with systemic lupus erythematosus. Proc. Nat. Acad. Sci. (US) 75:3464-3468, 1978.

3. Twomey, J.J., Laughter, A.H., and Steinberg, A.D.: A serum inhibitor of immune regulation in patients with systemic lupus erythematosus. J. Clin. Invest. 62:713-715, 1978.

4. Fauci, A.S., Steinberg, A.D., Haynes, B.F., and Whalen, G.: Immunoregulatory abberrations in systemic lupus erythematosus. J. Immunol. 121:1473-1479, 1978.

5. Pincus, T., Steinberg, A.D., Blacklow, N.R., and Decker, J.L.: Reactions with cellular and virus antigen preparations in sera of patients with systemic lupus erythematosus. Arthritis Rheum. 21:873-879, 1978.

6. Reinertsen, J.L., Klippel, J.H., Johnson, A.H., Steinberg, A.D., Decker, J.L., and Mann, D.L.: B-lymphocyte alloantigens associated with systemic lupus erythematosus. New Eng. J. Med. 299:515-518, 1978.

7. Moutsopoulos, H.M., Gallagher, J.D., Decker, J.L., and Steinberg, A.D.: Herpes zoster in patients with systemic lupus erythematosus. Arthritis Rheum. 21:798-802, 1978.

8. Steinberg, A.D., Klassen, L.W., Budman, D.R., and Williams, G.W.: Immunofluorescence studies of anti-T cell antibodies and T cells in systemic lupus erythematosus: Selective loss of brightly staining T cells in active disease. Arthritis Rheum. 22:114-122, 1979.

9. Pons, V.G., Reinertsen, J.L., Steinberg, A.D., and Dolin, R.: Decreased cell mediated cytotoxicity against virus-infected cells in systemic lupus erythematosus. J. Med. Virology (in press).1979.

10. Sakane, T., Steinberg, A.D., Arnett, F.C., Reinertsen, J.L., and Green, I.: Studies of immune functions of patients with systemic lupus erythematosus. III. Characterization of the lymphocyte subpopulations responsible for the defective autologous MLR. Arthritis Rheum. (in press). 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41025-08 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies on the cell surface receptor for IgE

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Metzger	Chief, Sec. Chemical Immunol.	ARB/NIAMDD
OTHER:	C. Isersky-Carter	Visiting Scientist	ARB/NIAMDD
	J. Kanellopoulos	Visiting Scientist	ARB/NIAMDD
	H. Hartmann	Staff Fellow	ARB/NIAMDD
	C. Fewtrell	Guest Worker	ARB/NIAMDD
	D. Holowka	Guest Worker	ARB/NIAMDD
	A. Goetze	Guest Worker	ARB/NIAMDD
	T. Triche	Head, Ultra Pathology	LP/NCI

COOPERATING UNITS (if any)
Department of Medicine, Johns Hopkins University (A. Sobotka and L. Lichtenstein)
Department of Pathology, University of Connecticut (E. Becker)

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Chemical Immunology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
10 1/4	8	2 1/4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are pursuing an in-depth investigation on the structure and function of the membrane receptor for IgE. For these studies intact cells from a rat basophilic leukemia as well as normal rat mast cells are being used. In addition receptor-bearing membrane vesicles and solubilized receptors are being studied. The aim is to understand the structure of the receptor as well as its integration in the plasma membrane. Results of such studies as well as direct functional studies are aimed at understanding the early stimulation of cells by antigen-induced, antibody-mediated reactions.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

IgE is a unique class of immunoglobulin which plays a predominant role in the physiological events associated with a variety of allergic phenomena. The immunoglobulin becomes attached to the surface of basophils and mast cells and, upon exposure to the allergenic antigen, mediates the release of histamine and other vasoactive amines from the cells to which it is attached. Although there is an increasing amount of information concerning the biochemistry of this reaction almost nothing is known about how the IgE acts as the cell surface transducer. The present project is directed towards increasing our understanding of how the IgE interacts with mast cells. In addition we wish to understand the mechanism by which the ternary complex of antigen-IgE antibody-cell surface receptor triggers the degranulation of basophilic cells.

Methods Employed:

No unusual new methods have been initiated during the past year.

Major Findings:

Since our previous report the following progress has been made:

A. Functional Studies:

1. Nature of triggering unit

We had previously developed data which indicated that (a) dimers of IgE served as a sufficient 'trigger' to generate unit signals (b) higher aggregates did not generate stronger signals. Recent results growing out of a collaboration with Drs. L. Lichtenstein and A. Sobotka at Johns Hopkins involving human basophils - promted us to re-examine this question. Chemically cross-linked IgE dimers, trimers and tetramers and higher polymers were isolated from preparations of human or rat IgE using dimethylsuberimidate. The results are indicated in the Table.

Relative Activity

	Human IgE	Rat IgE	
	blood basophils (*in vitro* release)	mast cells (skin test)	tumor basophils (*in vitro* release)
Monomer	0	0	0
Dimer	10-25	50	0.5
Trimer	50	100	15
Tetramer	100	100	100

These results, particularly those shown in the last column, suggest that higher aggregates can give stronger triggering signals than dimers (even though we have repeatedly shown that dimers are active). This observation places certain constraints on theories about the mechanism of recepetor function.

During the course of these studies it was found that D_2O could remarkably enhance the amount of degranulation observed with minimal signals. This finding (similar to that found with normal human and rodent cells) while unexplained will prove useful in future studies.

2. Studies are continuing on the nature of Ca^{++} fluxes during cell triggering. The stimulated efflux previously described by us can be shown (1) to require receptor cross-linking (2) not to occur when a variety of other surface proteins are cross-linked and (3) to occur on cells which do not degranulate. These findings suggest that a true early event is being monitored. It gives us a clear-cut function to look for in reconstitution studies. During the course of the studies we re-examined the use of ionophores to trigger the tumor cells. Previous studies failed completely to find conditions where ionophores would trigger non-cytotoxic release. We have now found that a small increase in pH had the desired effect. The finding has permitted corroboration of previous evidence that the defect in our non-secreting tumor cells appears to be at a stage later than that related to Ca^{++} fluxes.

3. We are continuing studies to see if the receptor becomes associated with other macromolecules during cell-activation using cross-linking reagents. So far all studies are negative. (See also Sec.B2)

4. Studies on the 'natural history' of the receptor are continuing. Studies with monomeric IgE showed that binding of the latter to the cell surface did not elicit changes. Studies with oligomers (capable of activating the degranulation process) are in progress.

B. Structure Studies

1. Membranes - We are now in a position to analyze critically the manner in which the receptor is integrated into the membrane. For such studies it will be useful to prepare inverted plasma membrane vesicles in order to analyze which portion (if any) of the receptor penetrates into the cytoplasm. Initial experiments to isolate such inside-out vesicles appear promising. Differential labeling studies with non-penetrable, lipid partioning and penetrable reagents have been begun.

2. Only slow progress has been made in regard to defining the component ("x") with which the receptor becomes cross-linked when either intact cells or detergent extract are treated with cross-linking reagents. A variety of technical problems has beset this project. Recently success has been achieved in intrinsically labeling the receptor with radioactive amino acids and suggestive evidence for incorporation into the elusive component "x" has been obtained. During the course of this study we have synthesized an iodinatable alkylating reagent N(chloroacetyl)iodotyramine. This can potentially be used to label unknown components generated by cleaving components cross-linked with disulfide containing cross-linking reagents.

3. Isolated receptor can now be prepared in substantial amounts. Compositional analysis has yielded a reproducible amino acid composition. The amino-terminus appears to be blocked. Further studies to define the carbohydrate content were temporarily thwarted when it was found that the gel matrix used during the final purification step was leaching large amounts of carbohydrate. We have now found that hydroxyapatite can be used as a final step which should obviate this problem.

4. Cynogen bromide cleavage of the purified receptor should be a useful first step in preparing peptides suitable for primary structure (sequence) analysis. Excellent progress has been made in analytical-scale experiments and preparative-scale experiments have begun.

Significance to Bio-Medical Research and the Program of the Institute:

The IgE mast cell system is of significance in the pathophysiology of allergy, parasitic infections, and inflammation. It is also an experimentally convenient system to study mechanisms of antigen-induced antibody-mediated cellular reactions in general. Finally it is an excellent model for the study of exocytosis. So far we are continuing to make good progress in the elucidation of the molecular mechanisms of this system. Knowledge of these may eventually permit rational intervention in situations where the system contributes to pathology.

Proposed Course:

1. Continue use of differential labeling studies to define nature of receptor integration into membrane.

2. Continue compositional analysis including carbohydrate and sulfhydryl analyses.

3. Continue peptide cleavage studies.

   (a) large-scale preparation of CNBr fragments for sequence analyses

   (b) analytical-scale studies using proteases

4. Continue functional studies
   (a) further definition of Ca^{++} fluxes
   (b) phosphorylation studies

5. Complete analysis with cross-linking reagents to define the component with which the receptor for IgE appears to be associated.

6. Begin reconstitution studies

Publications:

1. Isersky, C., Taurog, J.D., Poy, G., and Metzger, H.: Triggering of cultured tumor mast cells by antibodies to the receptor for IgE. J. Immunol. 121: 549-558, 1978.

2. Metzger, H.: The IgE-mast cell system as a paradigm for the study of antibody mechanisms. Immunological Rev. 41: 186-199, 1978.

3. Siraganian, R.P., and Metzger, H.: Evidence that the "mouse mastocytoma" cell line (MCT-1) is of rat origin. J. Immunol. 121: 2584-2585, 1978.

4. Metzger, H.: Early molecular events in antigen-antibody cell activation. Ann. Rev. Pharm. Toxicol. 19: 427-445, 1979.

5. Fewtrell, C., Kessler, A., and Metzger, H.: Comparative aspects of secretion from tumor and normal mast cells. Adv. Inflammation Research 1: 205-221, 1979.

6. Metzger, H.: Antibodies as Cell Surface Receptors. In DeLisi, C., and Blumenthal, R (Eds.): Physical Chemical Aspects of Cell Surface Events in Cellular Recognition. New York, Elsevier, 1979, pp. 237-241.

7. Kanellopoulos, J., Rossi, G., and Metzger, H.: Preparative isolation of the membrane receptor for immunoglobulin E. J. Biol. Chem. (in press) 1979.

8. Isersky, C., Rivera, J., Mims, S., and Triche, T.J.: The fate of IgE bound to rat basophilic leukemia cells. J. Immunol. 122:1926-1936, 1979.

9. Taurog, J.D., Fewtrell, C., and Becker, E.L.: IgE-mediated triggering of rat basophilic leukemia cells: Lack of evidence for serine esterase activation. J. Immunol. 122: 2150-2153, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41028-04 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Renal effects of aspirin and other non-steroidal anti-inflammatory drugs in SLE

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Plotz	Senior Investigator	ARB NIAMDD
OTHER:	J.R. Gill, Jr.	Chief, Hypertension and Endocrin. Br.	EB NHLI

COOPERATING UNITS (if any)
Hypertension and Endocrinology Branch, National Heart and Lung Institute

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/4	1 1/4	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Aspirin, an important drug in the therapy of many connective tissue diseases including systemic lupus erythematosus, has been found (Seaman, W.E., and Plotz, P.H.: Arth. Rheum. 19:155-160, 1976) to raise serum creatinine and BUN and lower creatinine clearance when administered to some patients with SLE as well as to some normal volunteers. Although this effect appears to be rapidly and completely reversible, if it is unrecognized it may falsely suggest deteriorating renal function due to activity of the underlying diseases. Our studies have shown that this effect is related to renal blood flow and tubular function, and that it correlates with renal output of prostaglandin. Other drugs besides aspirin which inhibit prostaglandin synthetase also cause these effects.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To investigate the mechanism of the apparent alteration in renal function induced by prostaglandin-synthetase-inhibiting non-steroidal anti-inflammatory drugs.

Methods Employed:

In patients requiring aspirin therapy, careful studies of renal function are performed. In selected patients with systemic lupus erythematosus, studies include inulin and PAH clearances to determine GFR, RBF, and tubular function.

Major Findings:

Aspirin and other non-steroidal anti-inflammatory drugs in therapeutic doses produces an acute fall in creatinine clearance in some patients with systemic lupus and in some normals.

Significance to Bio-Medical Research and the Program of the Institute:

Recognition of these effects should prevent misinterpretation of alterations in renal function in patients taking drugs that influence renal prostaglandin synthesis.

Proposed Course:

Observations will continue.

Publications:

1. Kimberly, R.P., Gill, J.R., Bowden, R.E., Keiser, H.R., and Plotz, P.H.: Elevated urinary prostaglandins and the effects of aspirin on renal function in lupus erythematosus. Ann. Int. Med. 89: 336-341, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41029-03 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Development of Computerized Clinical Database for Systemic Lupus Erythematosus (SLE)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.H. Klippel	Senior Investigator	ARB NIAMDD
OTHER:	J.L. Decker	Chief	ARB NIAMDD
	E.K. Harris	Chief	LAS DCRT
	G. Shakarji		DMB DCRT

COOPERATING UNITS (if any)
Laboratory of Applied Studies, DCRT
Data Management Branch, DCRT
Laboratory of Statistical and Mathematical Methodology, DCRT

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 2	OTHER: 0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The evaluation of long term randomized therapeutic studies of SLE would be facilitated by the development of a comprehensive clinical database. In this project, an ongoing computerized storage and retrieval system will be used to monitor clinical and therapeutic information on SLE patients participating in studies of immunosuppression and diffuse glomerulonephritis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Long term randomized trials comparing the efficacies of several different forms of immunosuppressive therapies and prednisone in the treatment of SLE diffuse glomerulonephritis have been in progress within our branch since 1970. These studies require careful monitoring of sequential observations of drug administration, clinical and laboratory studies and complications to determine the superiority of any single program. It is the objective of this project to develop a computerized data storage and retrieval system to monitor and evaluate these clinical trials.

Methods Employed:

A time oriented database will be used to record drug administration, clinical manifestations, studies of renal function, relevant laboratory studies and select complications of SLE patients participating in immunosuppressive studies. Information will be made current at the time of each inpatient, as well as outpatient, Clinical Center visits. The entry of data will be made from terminals located within the Clinical Center and datafile stored at DCRT.

Major Findings:

None at this time.

Significance to Bio-Medical Research and the Program of the Institute:

This project will benefit ARB clinical research activities by (1) facilitating the monitoring and assessment of therapeutic studies of SLE, (2) permitting analysis of interrelationships of multiple variables in the course of SLE and (3) improvement of the care of SLE patients participating in ARB studies.

Proposed Course:

The entry of data will be made both from retrospective information collected from patients involved in current trials and prospectively once a file has been established. Analyses of changes in renal function using a commulative summation technique will be used to compare the relative efficacies of different immunosuppressive therapies.

Publications:

None at this time.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41030-03 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Clearance of immune complexes by patients with systemic lupus erythematosus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Plotz	Senior Investigator	ARB NIAMDD
OTHER:	D. Finbloom	Clinical Associate	ARB NIAMDD
	D. Magilavy	Clinical Associate	ARB NIAMDD
	M. Frank	Chief, Lab. of Clin. Invest.	LCI NIAID
	T.J. Lawley	Investigator	DB NCI
	M. Hamburger	Clinical Associate	LCI NIAID

COOPERATING UNITS (if any)
Laboratory of Clinical Investigation, NIAID
Dermatology Branch, NCI

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4 1/2	4	1/2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clearance of IgG and IgM-coated autologous red cells is studied in patients with systemic lupus not taking corticosteroids. Abnormal clearance of IgG-coated cells has been found in some patients with active disease.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To determine whether altered clearance of immune complexes is pathogenetic in systemic lupus.

Methods Employed:

Autologous red cells are labeled with ^{51}Cr and coated with anti-blood group antibody. The cells are returned to the donor and samples drawn for several hours are tested for radioactivity.

Major Findings:

Clearance of IgE-coated RBC is abnormal in some patients with systemic lupus. Therapy leading to clinical improvement in associated with improvement in the clearance of complexes. Abnormalities also occur in some patients with rheumatoid arthritis.

Significance to Bio-Medical Research and the Program of the Institute:

This method allows a probe of RE clearance of immune complexes in a disease in which the presence of complexes is probably pathogenetic.

Proposed Course:

Work will continue.

Publications:

1. Frank, M.M., Hamburger, M.I., Lawley, T.J., Kimberly, R.P., and Plotz, P.H.: Defective reticuloendothelial system Fc-receptor function in systemic lupus erythematosus. N. Engl. J. Med. 300: 518-523, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41035-02 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Lymphocyte Depletion in the Treatment of Rheumatoid Arthritis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.L. Decker	Chief	ARB NIAMDD
	J. Karsh	Guest Worker	CIB NIDR
OTHER:	J.H. Klippel	Senior Investigator	ARB NIAMDD
	A.B. Deisseroth	Chief	POB NCI
	D.G. Wright	Senior Investigator	POB NCI
	W. Flye	Senior Investigator	SURG NCI

COOPERATING UNITS (if any)
Experimental Hematology Section, DCT NCI

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 1/2	2 1/4	1/4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project will evaluate the effect of removal of large numbers of peripheral blood mononuclear cells in patients with severe rheumatoid arthritis. Vascular access will be obtained by forearm arteriovenous fistula and lymphapheresis performed three times weekly for 10 weeks using a continuous flow cell separator. The removed cells will be studied for membrane characteristics and in vitro assays of immune function. The clinical activity of the arthritis will be quantitated by a global activity index during and following completion of the project.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Although the etiology of RA is not known, involvement of both the cellular and humoral immune systems are thought to play a basic role in the pathogenesis of this chronic systemic disorder. This is best evidenced by the favorable effect on RA of cytotoxic, or immunosuppressive, drugs and lymphocyte depletion by prolonged drainage of the thoracic duct. This project will evaluate the effect on RA of the removal of peripheral blood mononuclear cells using a continuous flow cell separator.

Methods Employed:

Patients with severe active RA unresponsive to conventional therapies will participate by informed consent. A temporary forearm side to side arteriovenous fistula will be constructed for purposes of obtaining repeated vascular access. Three times weekly lymphocyte removal using an Aminco continuous flow cell separator will be performed for a period not to exceed 10 weeks. The activity of the RA will be quantitated weekly using a standard global activity index. The frequency of lymphapheresis will be altered according to degrees of responsiveness. The removed cells will be quantitated and studied for lymphocyte subpopulations, surface membrane characteristics and *in vitro* immune function.

Major Findings:

Four patients with seropositive, erosive RA have been studied. In the course of 14 to 18 lymphapheresis procedures per patient, a mean of 11.1×10^{10} by lymphocytes and 2.1×10^{10} monocytes have been removed. This has resulted in a greater than 50% improvement (mean 68%) of the Articular Index in all patients and persisted for up to 19 weeks after discontinuation of lymphapheresis.

Significance to Bio-Medical Research and the Program of the Institute:

The removal of lymphocytes from the circulation should lead to an early diversion of cells from the inflammed synovium and an eventual depletion of select cell populations. The rate of reduction of synovial inflammation during lymphapheresis and its anticipated recurrence after discontinuing the procedure at 10 weeks will be observed to assess the potential therapeutic usefullness of this type of intervention.

The importance of circulating mononuclear cells, lymphocytes and monocytes, in chronic RA inflammation will be studied.

Proposed Course:

The pilot phase of the project evaluating lymphapheresis in RA patients has been completed and a report detailing the study findings is in preparation.

Publications:

1. Karsh, J., Wright, D.G., Klippel, J.H., Decker, J.L., Deisseroth, A.B., and Flye, M.W.: Lymphocyte depletion by continuous flow cell centrifugation in rheumatoid arthritis; clinical effects. Arth. Rheum. (in press), 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE (PROJECT NUMBER (Do NOT use this space))	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41036-02 ARB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Epidemiology of Canine and Human Systemic Lupus Erythematosus (SLE)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. H. Klippel	Senior Investigator	ARB NIAMDD
OTHER:	J.L. Decker	Chief	ARB NIAMDD
	A.D. Steinberg	Senior Investigator	ARB NIAMDD

COOPERATING UNITS (if any)

Arthritis and Immunologic Diseases Activity, Center for Disease Control, Atlanta, Georgia; St. Louis Park Medical Center, Minneapolis, Minnesota

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Disease

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/4	0	1/4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project will compare the incidence of clinical and serologic autoimmune abnormalities in members of households exposed to known cases of canine SLE or appropriately matched control dogs.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

This project will evaluate the relationship between canine and human SLE, by means of systematic epidemiologic survey, comparing households exposed to known cases of canine SLE with appropriately matched control households.

Methods Employed:

1. Approximately 20 dogs with recently diagnosed SLE or high titered antinuclear antibodies will be identified with the cooperation and assistance of regional veterinary centers specializing in the laboratory evaluation and study of canine SLE. Appropriate control dogs will be selected and matched to the SLE dog for defined household characteristics.

2. The head of each household in which the dogs reside will be contacted and permission for study obtained.

3. Each "SLE dog" and "control dog" household will be visited and a systematic epidemiologic survey completed. This will include a chronologic record of persons present in the household during the tenure of the dog, an estimate of the intensity of dog-human contact for all household members exposed to the dog, and a thorough review of medical histories of household members.

4. Blood specimens will be obtained from all household members and evaluated for antinucleic acid antibodies, antinuclear antibodies, rheumatoid factors, immunoglobulins and lymphocytotoxic antibodies.

5. The incidence of clinical and laboratory abnormalities in SLE and control dog households will be analyzed according to chronologic and contact relationships with the dogs.

Major Findings:

The project has studied 83 members of 19 households who were exposed to a dog with high titer antinuclear antibodies and 50 members of 16 control households matched for dog age, sex and veterinarian. No differences between these two populations have been identified with regard to clinical SLE symptoms or antinuclear or lymphocytotoxic antibodies, rheumatoid factors or serum immunoglobulins. Thus, the study has not detected any clinical or serologic effect of human exposure to canine SLE.

Significance to Bio-Medical Research and Programs of the Institute:

There is evidence to suggest that both environmental and genetic factors are important in human SLE pathogenesis. This project will attempt to evaluate the important environmental consideration of the potential relationship between dog and human SLE.

Proposed Course:

The data collection phase and preliminary analysis of the project have been completed; no additional households will be studied. A report detailing the findings of the project is in preparation.

Publications:

None at this time.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41037-02 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Genetic Factors in Systemic Lupus Erythematosus (SLE): Study of the Major Histocompatibility Complex

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.H. Klippel	Senior Investigator	ARB NIAMDD
	D.L. Mann	Senior Investigator	IB NCI
OTHER:	J.L. Decker	Chief	ARB NIAMDD
	A.D. Steinberg	Senior Investigator	ARB NIAMDD

COOPERATING UNITS (if any)

Immunology Branch, NCI

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The multiple occurrences of SLE over several generations in families suggest the importance of genetic factors in SLE pathogenesis. In this project, allo-antigens on B-lymphocytes, coded for by genes on the sixth chromosome known as the major histocompatibility complex, will be examined in patients with SLE. Select families with multiple occurrences of SLE will also be studied.

These alloantigens have physico-chemical and amino acid sequence homologies with murine B-cell antigens responsible for the regulation of specific immune responses. The frequency of individual alloantigens in SLE, as compared to normal controls, will be used in an attempt to identify immune genetic markers associated with SLE.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Specific cell surface antigens coded for by genes in the HLA-D region of the major histocompatibility complex have the potential to be associated with an increased susceptibility to autoimmune diseases. These human alloantigens can be detected on B-lymphocytes and monocytes and have physico-chemical and amino acid sequence homologies with murine B-cell antigens which regulate immune responses. These antigens can be identified by reactivity with B-lymphocyte alloantisera and, in certain instances, assignments of HLA-Dw specificities made on the basis of serologic reactivities.

In this project, we will test the hypothesis that specific HLA-D region genes are associated with SLE by determining B-lymphocyte alloantigens in lupus patients.

Methods Employed:

1. Lymphocytes from SLE patients will be obtained by Ficoll-Hypaque sedimentation and B-lymphocytes isolated by adherence to anti-human $F(ab)_2$ coated plastic plates.

2. B-lymphocytes will be typed by a complement-dependent microcytotoxicity assay using a serum panel from the Seventh International Histocompatibility Workshop (Oxford) and a second panel of 47 sera obtained from multiparous women.

3. HLA-DRw types will be assigned on the basis of reactivities of the Oxford Workshop panel.

4. The reaction frequencies of individual sera and HLA-DRw types will be compared between SLE patients and normal controls.

Major Findings:

1. B-lymphocyte alloantigens defined by the non-Workshop panel of 47 pregnancy sera have been compared in 53 patients with SLE and 184 normal controls. One serum, Ia-715, reacted with B-lymphocytes from 72% of SLE patients and 14% of controls ($p_c < 0.005$, relative risk 15.4).

2. HLA-DRw assignments using the Workshop panel have been compared in 39 SLE patients and 490 controls. Both HLA-DRw2 (51% vs 26%, $p_c < 0.004$) and HLA-DRw3 (49% vs 22%, $p_c < 0.004$) were increased in systemic lupus erythematosus.

3. Lymphocyte typing studies of seven families with multiple occurrences of SLE have suggested that the coinheritance of several B-lymphocyte alloantigens may influence lupus susceptibility.

In summary, this study demonstrates that select B-lymphocyte alloantigens, which are controlled by genes in the major histocompatibility complex, are present in increased frequency in SLE.

Significance to Bio-Medical Research and Programs of the Institute:

Immune response genes have been extensively studied in a variety of species. These genes are located within a gene grouping called the major histocompatibility complex and code for cell surface antigens found predominantly on B-lymphocytes and macrophages. These antigens play a major role in the regulation of specific immune responses.

Human immune response genes have yet to be fully characterized. However, human immune responses appear to be influenced by genes linked to the major histocompatibility. Furthermore, human B-lymphocyte alloantigens have been identified which are structurally similar to murine B-cell antigens.

Immune response genes may influence an individual's susceptibility to autoimmune diseases. This hypothesis will be evaluated by determining B-cell alloantigens, which are coded for by genes present in the region of presumed immune response genes, in patients with SLE.

Publications:

1. Reinertsen, J.L., Klippel, J.H., Johnson, A.H., Steinberg, A.D., Decker, J.L., and Mann, D.L.: B-lymphocyte alloantigens associated with systemic lupus erythematosus. N. Engl. J. Med. 299: 515-518, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41038-02 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Renal biopsy pathology in systemic lupus erythematosus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J.E. Balow	Senior Investigator	ARB/NIAMDD

COOPERATING UNITS (if any)
Armed Forces Institute of Pathology, Washington, D.C.
(T.A. Antonovych, M.D.)

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/4	1/4	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Detailed analysis of renal biopsy pathology has been undertaken in patients with systemic lupus erythematosus who are receiving various therapeutic regimens of immunosuppressive agents. Biopsies will be scored for various indices of pathological activity as well as for patterns of deposition of immune complexes by immunofluorescence and electron microscopy; these morphologic parameters are to be related to renal function data in order to ascertain whether more accurate prognostic indices can be developed and whether more sensitive measures of therapeutic response can be identified.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The classification of renal biopsy pathology in systemic lupus erythematosus (SLE) into general categories based mainly on the extent of cellular proliferation of glomerular cells as assessed by light microscopy has been a relatively poor predictor of prognosis in treated or untreated patients. Furthermore, it is currently recognized that certain cases of SLE nephritis may undergo transformation from one histologic class to another. The present study will examine the morphologic characteristics of SLE nephritis over time in patients being studied in ongoing therapeutic protocols. Biopsies will be scored for specific pathologic parameters which will be used as semi-quantitative indices of disease activity or chronicity. Repeat renal biopsy will be performed for evaluation of the same parameters at a specific time period, i.e. two years, regardless of the apparent clinical response of the patient to ascertain whether more accurate prognostic indicators can be identified and whether more sensitive measures of therapeutic response can be identified.

Methods Employed:

Percutaneous renal biopsy is performed under direct fluoroscopic visualization. Tissue samples are processed for routine light, immunofluorescence and electron microscopy. Sections are blinded and scored by both a nephropathologist and a clinical nephrologist on a checklist of specific morphological parameters.

Major Findings:

Clinical and routine laboratory studies may not identify patients with morphologically active disease as determined by renal biopsy.. Moreover, in those patients with clinically active renal disease, no specific parameter has been found to be predictive of the distribution nor the severity of pathologic lesions. We have found that patients with renal biopsies having a classification of diffuse proliferative glomerulonephritis have a broad spectrum of severity as judged by the type and number of active lesions either within glomeruli or in the interstitium. In addition the significance of sclerosing lesions, which may be concomitantly present with proliferation, appears to be an important prognostic factor which has not received adequate consideration in most series. Studies of serial renal biopsies using the semi-quantitative histological scoring system have been undertaken in patients who have entered a chronic stable phase of lupus nephritis. Preliminary results suggest that renal histologic changes are more severe in patients receiving corticosteroids alone compared to those receiving cytotoxic agents in addition. Analysis of larger numbers of patients is actively underway.

Significance to Bio-Medical Research and the Program of the Institute:

An improved system for evaluation and classification of renal biopsy pathology would provide the following advantages:

1. A clearer description of the types of pathology in SLE nephritis.

2. The ability to select more homogeneous patients for study of immunologic, renal function and therapeutic responses.

3. Improved ability to identify accurate prognostic indicators of subsequent renal function.

4. Improved bases of indications for initiating and terminating therapy for SLE nephritis.

Proposed Course:

An ongoing study of patients who will be asked permission for repeat renal biopsy every two years is planned. Patients will be studied as they enter new therapeutic protocols as well as those followed in previous treatment programs.

Publications:

1. Balow, J.E.: Clinicopathological correlations in lupus nephritis, In Decker, J.L. (Moderator); Systemic lupus erythematosus: Evolving concepts. Ann. Int. Med. (in press) 1979.

2. Balow, J.E., and Klippel, J.H.: Effects of immunosuppressive therapy on the histologic evolution of lupus nephritis. In Schreiner, G.E. (Ed.): Controversies in Nephrology - 1979. Washington, Georgetown Press, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41039-02 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Regulation of cell mediated immune responses in normals and patients receiving immunosuppressive therapy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.E. Balow	Senior Investigator	ARB NIAMDD
OTHER:	C.J. Derr	Biologist	CC

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/4	1 1/4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of various immunosuppressive agents on lymphocyte functions is being studied to identify their sites and mechanisms of action. Various agents, such as cyclophosphamide and azathioprine are also being used as tools to study cell-mediated immune regulation in normal lymphocytes, as well as those from patients with autoimmune disorders, particularly SLE. The major goals of these studies are to further eludicate the mechanisms of altered immunoregulation, as well as to find new parameters of immune function to measure and/or monitor responses of patients receiving pharmacologic immunosuppressions.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Abnormalities of immunoregulation are suspected to be the cause of several autoimmune disorders. Although some features of the complex cellular interactions involved in regulatory processes have been identified in normal subjects, studies of subjects with specific disorders and those receiving chemical immunosuppression have yielded important breakthroughs in our understanding of cells mediated immune regulation. The present studies will examine changes in immunologic function induced by immunosuppressive agents as well as by new experimental techniques.

Methods Employed:

1) Cell mediated cytotoxicity and lymphocyte proliferation assays will be predominantly used to evaluate the differential effect of cyclophosphamide on various subpopulations of lymphocytes from normal and patient donors. These studies will be performed by direct in vitro manipulation of lymphoid cells as well as by studies of changes induced by in vivo administration of these agents to patients with autoimmune disorders.

2) Studies of the role of circulating immune complexes in patients with autoimmune disease will be carried out. These will include studies of their role in the pathogenesis of glomerulonephritis, the effect of their removal by physical or chemical means, abnormalities of their clearance from the circulation in disease states (especially in glomerulonephritis), and studies of their possible effect on normal cell mediated immune responses.

Major Findings:

The in vivo administration of cyclophosphamide has been shown to augment T-lymphocyte function under certain conditions. Preliminary studies failed to show a differential sensitivity of immune regulator (suppressor) cells directly exposed to cyclophosphamide in vitro (Clin. Res. 27: 321A, 1979). These studies suggest that the enhancement of cell-mediated responses after in vivo cyclophosphamide results from a mechanism other than a direct inhibition of suppressor cells, which is under further investigation.

Significance to Bio-Medical Research and the Program of the Institute:

Characterization of the specific sites and mechanisms of action by various immunosuppressive agents would allow a greater selectivity in their clinical use for conditions associated with specific immunological defects. In addition, identification of their major site of action will hopefully lead to improvements in methods to monitor the desired effectiveness as well as the potential toxicity of these agents. Development of more selective means for correction of immunological abnormalities would obviously greatly enhance our care of clinical disorders.

Proposed Course:

Long term studies of both patients and normals will be undertaken.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41040-07 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Study of various cytotoxic drug programs in diffuse lupus nephritis (PACIV)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.D. Steinberg	Senior Investigator	ARB NIAMDD
	J.L. Decker	Chief, Arthritis and Rheumatism Br.	ARB NIAMDD
OTHER:	P.H. Plotz	Senior Investigator	ARB NIAMDD
	J.H. Klippel	Senior Investigator	ARB NIAMDD
	J.E. Balow	Senior Investigator	ARB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	4 1/4	3/4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with the diffuse forms of systemic lupus erythematosus nephritis are invited to enter a therapeutic trial. After modification of the plan in the course of the work, the three programs to which admissions continue are corticosteroids only, corticosteroids with cyclophosphamide and azathioprine both in low doses, and corticosteroids with intermittent intravenous cyclophosphamide.

Regular follow-up of the 55 patients admitted continues.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The Arthritis and Rheumatism Branch has a major interest in the management of the nephritis of systemic lupus erythematosus (SLE). Renal failure is the most common cause of death in the disease. Deterioration of renal function, in some gradually and in others precipitately, does not seem to be much altered by adrenal corticosteroid therapy, a form of treatment which does control most of the symptomatic features. The renal damage is produced by immune complex deposition in the glomeruli. It has been suggested that drugs capable of altering the antibody response, "immunosuppressives," would reduce the formation of antibody and thus of antigen-antibody complexes. The objective of this study is to assess the efficacy and toxicity of various immunosuppressive drug programs in SLE nephritis.

Methods Employed:

Patients with systemic lupus erythematosus and active nephritis will be invited to enter the trial. All patients are to be on prednisone and one group will receive no additional medications. The other four groups will receive in addition: Oral cyclophosphamide up to 3 mg/kg/day; oral azathioprine up to 3 mg/kg/day; oral cyclophosphamide up to 1 mg/kg/day and azathioprine up to 1 mg/kg/day; and every 3 months intravenous infusions of cyclophosphamide up to 1 gm/M^2. All patients are repeatedly reevaluated with a battery of renal function tests, serological studies, urinalyses and, after two years in the trial, by renal biopsy when possible. It is proposed to continue the treatment programs indefinitely.

Major Findings:

The first patient was admitted in March 1973. By March 1976 (after the admission of 26 patients) the results of an earlier trial (CAP, Z01 AM 41000-10) showed that there was little if any advantage to the simple addition of oral cyclophosphamide or azathioprine to the usualy prednisone dose. Accordingly at that time the study was changed dropping 2 of the drug schedules and continuing to admit patients to the other 3 - prednisone only, combined cyclophosphamide and azathioprine by mouth, or intermittent intravenous cyclophosphamide.

To date (June 1979) 29 more patients to a total of 55 have been admitted and are under care.

The most recent complete review of this and the earlier study has been reported. It showed again no advantage to the addition of single oral agents; 55 to 60% of patients assigned to prednisone only, oral cyclophosphamide, or oral azathioprine were identified as stable or improved as compared to their admission status. However, 80 to 90% of those assigned to combined oral drug or intermittent intravenous cyclophosphamide were stable or improved, albeit at shorter follow-up interval.

The continued failure of the oral drugs to modify the disease and the appearance among our patients of two cases of carcinoma of the urinary bladder after oral cyclophosphamide administration has resulted in the addition of a plan for withdrawal of the single oral drugs; this is being done with suitable consideration of disease activity and appropriate evaluation as the individuals come in for follow-up.

Significance to Bio-Medical Research and the Program of the Institute:

Thse studies are relied upon by physicians concerned with SLE all over the world. We have been one of very few units able to speak from the hard data base of controlled studies. The net effect has been a great reduction in unwarranted enthusiasm for these agents. These studies certainly show that other modes of alleviating SLE renal disease are needed.

Proposd Course:

We will continue to follow these patients on their assigned programs.

Publications:

1. Klippel, J.H.: Studies in the treatment of lupus nephritis, in Decker, J.L. (Moderator): Systemic lupus erythematosus; evolving concepts. Ann. Intern. Med. (In press), 1979.

2. Plotz, P.H., Klippel, J.H., Decker, J.L., Grauman, D., Wolff, B., Brown, B.C., and Rutt, G.: Bladder complications in patients receiving cyclophosphamide for systemic lupus erythematosus or rheumatoid arthritis. Ann. Intern. Med. (In press), 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 41041-01 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Aspirin and ibuprofen in SLE

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Plotz	Senior Investigator	ARB NIAMDD
OTHER:	D. Magilavy	Clinical Associate	ARB NIAMDD
	D. Finbloom	Clinical Associate	ARB NIAMDD
	J.L. Decker	Chief, Arthritis and Rheumatism Br.	ARB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	3/4	1/4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Treatment of the non-life-threatening manifestation of systemic lupus erythematosus is often aspirin or another non-steroidal anti-inflammatory drug. This study compares the efficacy and toxicity of aspirin and ibuprofen in some mild manifestation of SLE.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To compare the efficacy and toxicity of aspirin and ibuprofen for managing some manifestation of SLE.

Methods Employed:

After a brief control period, patients are given aspirin or ibuprofen in a double-blind fashion and their clinical response and laboratory tests are observed.

Major Findings:

None yet.

Significance to Bio-Medical Research and the Program of the Institute:

This study is one of several carried out by the Arthritis and Rheumatism Branch to improve the management of SLE.

Proposed Course:

Patients will continue to be entered in this coming year.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41042-01 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Intravenous Cyclophosphamide in the Treatment of Rheumatoid Arthritis (RA)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.H. Klippel	Senior Investigator	ARB NIAMDD
OTHER:	J.L. Decker	Chief	ARB NIAMDD
	J. Karsh	Guest Worker	CIB NIDR
	P.H. Plotz	Senior Investigator	ARB NIAMDD
	N.L. Gerber	Chief	REHAB CC

COOPERATING UNITS (if any)
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Disease

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	1 1/4	1/4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The efficacy of intermittent intravenous cyclophosphamide in the treatment of rheumatoid arthritis will be evaluated in a double-blind randomized study. Patients unresponsive to conventional therapies will be assigned to receive a total of three intravenous infusions of either cyclophosphamide (1.0 gram per square meters, or less) or saline; one infusion every two months for a period of six months. The clinical activity of the arthritis and a functional assessment of patients will be quantitated during the study. Comparisons of the observed changes in these measures from assessments at the time of trial entry and two months after the final infusion will be made between treatment groups.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

A number of controlled clinical trials have convincingly demonstrated the effectiveness of cyclophosphamide in the treatment of rheumatoid arthritis. However, the major factors limiting the clinical use of the drug in the treatment of RA is drug toxicity, including bone marrow depression, alopecia, amenorrhea or azospermia, bladder complications, infection and the potential for neoplasia.

Our experience with the use of intermittant, intravenous cyclophosphamide in the treatment of systemic lupus erythematosus suggests that this mode of drug administration may provide effective immunosuppression with minimal drug complications.

In this project, we propose to evaluate the efficacy and toxicity of intravenous cyclophosphamide given every two months in the treatment of patients with rheumatoid arthritis.

Methods Employed:

1. Patients with "active" rheumatoid arthritis unresponsive to conventional therapies will be randomized to receive three intravenous infusions of either cyclophosphamide or saline; one infusion every two months for a period of six months.

2. The clinical activity of the arthritis will be quantitated with an Articular Index measuring joint pain, tenderness and swelling.

Major Findings:

None at this time.

Significance to Bio-Medical Research and the Program of the Institute:

Cyclophosphamide is, perhaps, the most effective drug available for the treatment of rheumatoid arthritis. However, the drug toxicity associated with oral therapy is a major problem and limits drug administration.

In this project, we will attempt to determine the clinical efficacy in RA of presumed safer mose of administration of cyclophosphamide.

Proposed Course:

Patients satisfying the study adminssion criteria are being actively recruited.

Publications:

None at this time.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41043-01 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Immunization of Patients with Systemic Lupus Erythematosus (SLE) with Pneumococcal Polysaccharide Vaccine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.H. Klippel	Senior Investigator	ARB NIAMDD
OTHER:	J. Karsh	Guest Worker	CIB NIDR
	N.I. Stahl	Clinical Associate	ARB NIAMDD
	A.D. Steinberg	Senior Investigator	ARB NIAMDD
	J.L. Decker	Chief	ARB NIAMDD

COOPERATING UNITS (if any)
Department of Microbiology and Immunology, Downstate Medical Center, Brooklyn, New York (G. Schiffman, Ph.D.)

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Diseases

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1 1/4	PROFESSIONAL: 1 1/4	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The immunogenicity and potential for disease modification of pneumococcal polysaccharide vaccine (Pneumovax) in SLE will be evaluated in a controlled, double-blind study. Patients will receive a single intramuscular dose of either vaccine or saline placebo. Anti-pneumococcal antibody concentrations to 12 type-specific pneumococcal capsular antigens will be measured by a radioimmune assay prior to and one and six months following vaccination. The patients will be monitored for clinical and laboratory abnormalities of SLE by a composite clinical, laboratory and serologic index.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Infections are a major cause of morbidity and mortality in patients with systemic lupus erythematosus. The protection afforded by immunizations might be of benefit in this population provided that vaccines are immunogenic without resulting in exacerbations of the underlying disease.

In this project, we will evaluate the immunogenicity and clinical and serologic effects of a multivalent pneumococcal polysaccharide vaccine in SLE in a controlled, randomized study.

Methods Employed:

1. Patients will be randomized to receive 0.5 ml of intramuscular polyvalent pneumococcal polysaccharide vaccine or isotonic saline solution containing 0.25% phenol.

2. Serum will be collected prior to and one and six months after vaccination. Antibodies to each of 12 capsular polysaccharide types, measured by a radioimmunoassay, will be compared in vaccine and placebo-treated patients.

3. Clinical and laboratory measures of SLE activity will be monitored prior to and one and six months post vaccination to determine the effect of immunization on the underlying disease.

Major Findings:

1. One month following vaccination, vaccine-treated patients demonstrated significant increases in antibody concentration to each of 12 type specific capsular antigens as compared to placebo-treated patients.

2. Both pre- and post-vaccine antipneumococcal antibody concentrations in SLE patients were less than levels in normal healthy controls.

3. No clinical, laboratory or serologic effects on the underlying SLE were observed at one month following vaccination.

Significance to Bio-Medical Research and Program of the Institute:

In spite of effective antibiotic treatment, infections continue to be a major cause of mortality in patients with SLE. Thus, preventative measures, in particular the benefits achieved through immunizations, are of particular importance in this patient population. This project will evaluate the immunogenicity of a multivalent, bacterial vaccine in SLE.

Proposed Course:

All study patients will be followed for a period of six months following vaccination to determine if post-vaccination increases in antibody concentrations are maintained and if late complications become evident. An attempt will be made to determine whether observed differences in antibody responsiveness to specific antigens is on the basis of SLE disease activity, medications or HLA genetics.

Publications:

None at this time.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 41044-01 ARB

PERIOD COVERED

October 1, 1978 to September 31, 1979

TITLE OF PROJECT (30 characters or less)

Lymphocyte Depletion vs Plasma Removal in the Treatment of Rheumatoid Arthritis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.L. Decker	Chief	ARB NIAMDD
OTHER:	J.H. Klippel	Senior Investigator	ARB NIAMDD
	J. Karsh	Guest Worker	CIB NIDR
	D.G. Wright	Senior Investigator	POB NCI
	A.B. Deisseroth	Chief	POB NCI
	W. Flye	Senior Investigator	SURG NCI

COOPERATING UNITS (if any)

Clinical Immunology Branch, NIDR; Pediatric Oncology Branch, NCI; Surgery, NCI

LAB/BRANCH

Arthritis and Rheumatism Branch

SECTION

Connective Tissue Diseases

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 1/2	2 1/4	1/4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with rheumatoid arthritis unresponsive to conventional therapies will be offered participation in a double-blind therapeutic trial. They will be randomized to two treatment protocols comparing the effects of lymphocyte depletion (lymphapheresis) and plasma removal (plasmapheresis) on rheumatoid synovitis. The disease activity will be monitored by an Articular Index. The risks and benefits of each treatment will be evaluated and compared at the end of 5 weeks of therapy and through a 6 to 12 month follow-up period.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

A previous pilot study (Clinical Project 76-A-25) suggested that lymphocyte depletion may benefit patients with severe rheumatoid arthritis. The process of lymphocyte depletion (lymphapheresis) involved not only the removal of lymphocytes but plasma, polymorphonuclear cells and platelets as well. Although circulating levels of serum albumin, polymorphonuclear cells and platelets did not change it is possible that temporary removal of serum factors and/or the above cells may have accounted for the beneficial effects. The goals of the study are to compare the relative benefits of cell depletion and plasma removal under identical circumstances.

Methods Employed:

Eight patients meeting predefined criteria for severe recalcitrant rheumatoid arthritis will be admitted to the trial and randomly assigned to either lymphapheresis or plasmapheresis. To ensure repetitive venous access arteriovenous fistulae will be constructed at the wrist. Upon fistula maturation patients will be re-admitted to undergo 15 procedures over a 5 to 6 week study period. Entry medications will be limited to non-steroidal anti-inflammatory drugs and prednisone and will be held constant for the preceding month, the period of treatment, and the follow-up period until relapse has occurred. A single examinor unaware of the nature of the patient's therapy will perform serial assessments of rheumatoid activity. Routine hematological, chemical and serological parameters will be followed. In addition special studies will follow the peripheral lymphocyte count and the distribution of lymphocyte sub-types in the circulation.

Major Findings:

To date, 4 patients have entered the study. A total of 60 procedures have been performed without a single adverse effect. Conclusions as to outcome of the above patients cannot be discussed at this time of writing.

Significance to Bio-Medical Research and Program of the Institute:

The confirmation of the beneficial effects of cell depletion and/or plasma removal will provide significant insights into the pathogenesis of rheumatoid arthritis. The procedures may provide new therapies for the rheumatoid patient who has failed on conventional medications and avoid the direct toxicities of cytotoxic medications.

Proposed Course:

Long-term follow up of the patients treated to date and the completion of the study will be accomplished.

Publications:

None at this time.

METABOLIC DISEASES BRANCH

The general goals of the Branch are to investigate the structure, secretion and biochemical mechanism of action of hormones governing ion transport and mineral metabolism and to apply the knowledge gained to understanding and treating related clinical disorders. Current investigations include hormone-receptor interaction, mechanism of receptor control of adenylate cyclase, cyclic AMP control of ion transport, and regulation of parathyroid hormone secretion. Systems under study include β-adrenergic receptors in several tissue, isolated parathyroid cell systems and skeletal and renal tissue. Clinical studies on hyperparathyroidism have utilized extensively research findings from the laboratory. Radioimmunoassays for parathyroid hormone have been applied to determine the hormone in samples obtained selectively by venous catheterization, thereby allowing localization of parathyroid adenomas. Further studies utilize urinary cyclic AMP clearance as a clinical parameter of parathyroid hormone action. Another investigation takes advantage of the autologous parathyroid transplant program developed in collaboration with the Surgery Branch, NCI. Autologous transplants to the arm allow direct sampling of draining veins for parathyroid hormone secretion in man under conditions wherein the tissue can be selectively challenged with potential secretagogues or inhibitors of parathyroid secretion.

β-Adrenergic Receptor of the Avian Erythrocytes.

Interactions of catecholamines with β-adrenergic receptors and activation of adenylate cyclase are under study with the plasma membranes of turkey erythrocytes and other cell systems. Specific receptors have now been identified on turkey erythrocytes, parathyroid cells, pineal cells, rat, guinea pig and monkey lung membrane preparations and rat liver membranes.

β-Adrenergic receptor sites are identified using a radioiodinated β-blocker iodo-hydroxybenzylpindolol. A series of β-adrenergic agonists and inhibitors compete for binding with affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. Receptor occupancy for agonists correlate linearly with function (cyclic AMP production). The stereospecificity of interaction with the iodinated β-blocking agent and the correspondence between affinity for site and biological effectiveness indicate that binding of iodohydroxybenzylpindolol validly reflects interaction with the β-adrenergic receptor.

Calcitonin receptors have been identified and studied in several tissues wherein they control generation of cyclic AMP-mediated processes. Although calcitonin polypeptides have undergone remarkable evolutionary changes in structure and effectiveness, there has been no significant corresponding change in biological nature of calcitonin receptors.
[Drs. Marx and Aurbach, NIAMDD]

Purine nucleotides enhance catecholamine-stimulated adenylate cyclase activity in the erythrocyte membrane. A guanine nucleotide binding protein activity from the catalytic unit regulates adenylate cyclase activity. Although guanine nucleotides (the most effective is guanylylimidodiphosphate, Gpp(NH)p) enhance isoproterenol-activated adenylate cyclase with apparent increase in sensitivity to the agonist, there is nevertheless no change in affinity of the receptor.

Treatment of turkey erythrocyte membranes with detergent solutions under appropriate conditions allows solubilization of adenylate cyclase (AC) in high yield. The enzyme activity is stabilized by incubation of membranes, prior to solubilization, with hormone (isoproterenol) and a guanine nucleotide (GN) such as Gpp(NH)p or 5'-GMP. The persistently active "holoenzyme" formed during treatment with Gpp(NH)p can be partially purified using conventional techniques (8-12 fold) and has an apparent molecular weight, in nonionic detergent solutions of 400,000-500,000 daltons. Incubation with GMP allows later identification and manipulation of the guanine nucleotide regulatory protein. Recently this guanine nucleotide regulatory unit has been isolated from the turkey erythrocyte adenylate cyclase complex with GTP affinity chromatography. The affinity-purified regulatory unit from turkey erythrocytes restores both GN and fluoride-responsive AC activity to a mutant mouse lymphoma cell line ($S49\ CYC^-$). Moreover, changes in the nature of the guanine nucleotide bound to the regulatory unit influence the ability of fluoride to maximally stimulate adenylate cyclase activity.
[Drs. Spiegel, Downs, Attie, Brown and Aurbach, NIAMDD]

Clinical studies are continuing on primary hyperparathyroidism and its familial variants. Detailed family screening and case finding has produced 34 kindreds for analysis. These studies have allowed segregation of the familial variants into two apparently distinct disease syndromes - familial multiple endocrine adenomatosis (MEN I) and familial hypocalciuric hypercalcemia (FHH). FHH was distinguished from MEN I by 1) a higher incidence of hypercalcemia below the age of 20 and virtually a 100% penetrance for hypercalcemia after age 20, 2) milder clinical manifestations - low incidence of recurrent nephrolithiasis or recurrent peptic ulceration, 3) no hypercalciuria, 4) normal basal concentrations of gastrin, and 5) poor response to subtotal parathyroidectomy. Distinction between the two syndromes, both inherited as autosomal dominant traits, is important because in FHH the clinical course is generally milder and subtotal parathyroidectomy is less likely to be beneficial. In FHH the ionized and ultrafiltrable calcium concentration in serum is elevated in proportion to the increase in total calcium. These patients have a high filtrable load of calcium associated with a marked decrease in renal calcium clearance. Plasma PTH concentration is lower in patients with FHH than in patients with similar degrees of hypercalcemia from typical primary hyperparathyroidism whether assessed by PTH radioimmunoassay or by renal clearance of cAMP or phosphate.
[Drs. Marx, Spiegel, Downs, Attie, Brown, Stock and Aurbach]

Studies on modes of localizing parathyroid tumors continue. A high degree of success has been obtained in localizing tumors through vascular catheterization procedures. Parathyroid arteriography developed and performed by Dr. John Doppman afforded, in approximately 45% of cases in which it was used, the identification of abnormal masses of tissue subsequently identified as parathyroid. Confirmation of the mass thus identified as parathyroid tissue can be accomplished by verifying high concentrations of parathyroid hormone by radioimmunoassay in veins draining the lesion. Draining vessels contain concentrations of hormone 2- to 20-fold or more greater than the concentration of parathyroid hormone detected by immunoassay in the peripheral plasma. In cases with single adenomas, this concentration

ratio is greatest selectively in the one vein draining the lesion. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by non-parathyroid cancers. Localization of anterior mediastinal parathyroid adenomas has become possible using computed tomography. Eight mediastinal adenomas have been treated nonsurgically by percutaneous catheter injection of occlusive agents into the arterial blood supply with 7 complete and one partial remissions.
[Drs. Marx, Spiegel, Downs, Attie, Brown, and Aurbach, NIAMDD: Dr. Doppman, Radiology Dept., C.C.]

The role of 1,25-$(OH)_2$-D_3, the most potent natural metabolite of cholecalciferol, has been assessed in hypocalcemic states. We have discovered a family with a unique disturbance of calciferol metabolism. Two siblings have rickets and osteomalacia attributable to decreased target tissue sensitivity to 1,25-$(OH)_2$-D. When their serum concentrations of 1,25-$(OH)_2$-D are maintained by various calciferol analogs at concentrations approximately 2.0-fold normal, their disorder can be fully controlled.
[Drs. Marx, Spiegel, Brown, Gardner, Downs, Attie, Hamstra and Deluca]

Determination of intraoperative urinary cAMP excretion in patients undergoing neck exploration for primary hyperparathyroidism has shown that succcessful removal of abnormal parathyroid tissue is followed by a rapid decline to low levels (<1.5 μmol/g creatinine). In patients in whom surgery was not curative, levels remained elevated. These results suggest that monitoring UcAMP excretion intraoperatively (utilizing techniques for rapid radioimmunoassay of cAMP) could aid in operative management of patients with primary hyperparathyroidism.

Determination of urinary cAMP excretion postoperatively in patients undergoing neck exploration for primary hyperparathyroidism is a useful method for assessing postoperative parathyroid function. UcAMP excretion declines postoperatively in all patients in whom hypercalcemia is corrected but not in those with persistent hypercalcemia. In patients becoming severely hypocalcemic (and requiring vitamin D therapy) postoperatively, UcAMP measurement enables one to distinguish patients with decreased parathyroid reserve as the cause for hypocalcemia (low UcAMP excretion) from patients with healing osteitis fibrosa ("hungry bones") as the basis for hypercalcemia. UcAMP in the latter group is often elevated but can be suppressed if serum calcium is normalized. Elevated UcAMP excretion postoperatively in the face of hypocalcemia enables one to predict that vitamin D therapy will be required temporarily and precludes the need for parathyroid autografts.
[Drs. Spiegel, Marx, Downs, Attie, Brown and Aurbach, NIAMDD]

Studies have been continued with dispersed bovine and human parathyroid cells prepared by collagenase and DNase digestion. Bovine cells are stimulated to secrete hormone by β-adrenergic catecholamines, dopamine, PGE_2, secretin and cholera toxin, while they are inhibited by calcium, magnesium, α-adrenergic catecholamines, $PGF_{2\alpha}$ and nitroprusside. In the case of agonists, there is a close correlation between stimulation of cAMP accumulation and enhanced

secretion. Antagonists inhibit cAMP accumulation and secretion, but the inhibitory effects of calcium are not explained quantitatively by decreases in cAMP content. Pathologic human parathyroid tissue differs from bovine tissue in its response to both calcium and secretagogues. Cells prepared from glands of patients with primary hyperplasia are inhibited by calcium in many cases in a normal or near normal fashion. Cells from adenoma, on the other hand, are either nonsuppressible or are inhibited by higher than normal calcium concentrations. Cells from pathologic glands are stimulated by β-adrenergic catecholamines, glucagon, PGE_2 and histamine but not by secretin or dopamine. Human cells also appear to lack the inhibitory receptors for α-adrenergic catecholamines and $PGF_{2\alpha}$.
[Drs. Brown, Gardner, Brennan and Attie]

Studies using hypotonic lysates of dispersed bovine oarathyroid cells have shown that dopamine can stimulate adenylate cyclase. The sensitivity to dopaminergic agonists and antagonists of the cyclase and intact cells (cyclic AMP accumulation and PTH release) were virtually identical suggesting that dopamine stimulated PTH release is mediated through stimulation of adenylate cyclase. Further studies have shown the specificity of the receptor to be similar to other recently described dopamine-sensitive cyclases (D-1 receptors; D-2 receptors do not lead to stimulation of cyclase).
[Drs. Brown, Gardner and Attie, NIAMDD]

The cooperative study between investigators in NIAMDD and members of the staff of Hadassah Medical School-Hebrew University involving approximately 300 ambulatory patients with osteoporosis in Jerusalem is now in its sixth year. By a combination of dietary survey, X-ray, bone mineral content, medical history and other methods, considerable information on the natural history of osteoporosis is anticipated; in addition, a double-blind study is being made of the possible value of oral administration of high calcium, high phosphate and fluoride in treatment of osteoporosis. At the dosage of fluoride being used, 20 mg F per day, undesirable side effects have been very low. Although the number of patients either medically unfit for the study or unwilling to cooperate has been much higher than expected, once entered into active study the drop-out rate has been moderate. Accession of new patients will be stopped this year. This study is also in the process of adding certain new techniques for evaluation of physiologic factors affecting bone metabolism: intestinal absorption tests (^{47}Ca), blood levels of a variety of vitamin D metabolites and fluoride tolerance tests. In addition, in association with this project members of the Physics Department, Hadassah Medical Organization, are being supported in the development of a relatively new technique of bone densitometry, Compton scattering, which it is hoped may be applied to the spine, a most difficult location in which to make such measurements, yet the most critical for assessment of the status of osteoporosis. [Dr. P. H. Bennett, Dr. G. D. Whedon]

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 43001-14 MD

PERIOD COVERED

October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies in Bone Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
G. D. Whedon, M.D., Director, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
A. M. Spiegel, M.D., Senior Investigator, MDB, NIAMDD
E. M. Brown, M.D., Senior Clinical Associate, MDB, NIAMDD
J. Shapiro, M.D., Associate Director, Clinical Center, NIH.

COOPERATING UNITS (if any)

Osteoporosis and Fracture Research Institute, Hadassah Medical Organization, Jerusalem, Israel.

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.25	.25	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of the project on bone metabolism are to investigate the factors influencing mineral storage and loss in demineralizing bone diseases and to study the influence of added calcium and phosphate on resorption of bone mineral. The project involves estimation of bone density by photon absorptiometry and various techniques relative to vitamin D and fluoride metabolism.

During the past year, clinical studies of osteoporosis have been continued, using photon absorption densitometry, with observations of the bone mineral content in various populations and of the possible effect thereon of dietary manipulation.

PHS-6040
(Rev. 10-76)

Objectives: To investigate the factors influencing mineral storage and loss in demineralizing bone diseases.

Methods Employed: (1) Estimation of bone density in the radius by photon absorptiometry. (2) Estimation of intestinal absorption of calcium by oral administration of tracer doses of ^{47}Ca. (3) Tests of fluoride tolerance.

Major Findings: Studies have continued in order to evaluate the clinical usefulness of photon absorption method of bone mineral analysis. Using this technique, with an iodine-125 source, values for bone mineral content in a normal population of children and adults were determined. These studies demonstrated a fairly linear increase in bone mineral content from birth into the third decade; thereafter bone mineral decreased in both men and women, but at a faster rate in women than in men.

Measurement of bone mineral content in the radius in black populations from birth through adult life disclosed a greater mineral content in blacks compared with whites at all ages. Based on group data (single or few measurements each on many individuals), black females lose mineral in the radius with aging. These studies confirm the clinical impression that osteoporosis is very uncommon in blacks and suggests that as currently defined osteoporosis should not occur as a primary disease in black males. Bone mineral content was determined in black patients with sickle cell anemia. Sixty-four percent of males and 39 percent of females with sickle cell disease have less bone mineral than normal black children of the same age. The ratio of bone mineral content to bone width followed the normal pattern in these children. Loss of bone mineral content did not correlate with severity of anemia or transfusion requirement.

The cooperative study between investigators in NIAMDD and members of the staff of Hadassah Medical School-Hebrew University involving approximately 300 ambulatory patients with osteoporosis in Jerusalem is now in its sixth year. By a combination of dietary survey, x-ray, bone mineral content, medical history and other methods, considerable information on the natural history of osteoporosis is anticipated; in addition, a double-blind study is being made of the possible value of oral administration of high calcium, high phosphate and fluoride in treatment of osteoporosis. At the dosage of fluoride being used, 20 mg F per day, undesirable side effects have been very low. Although the number of patients either medically unfit for the study or unwilling to cooperate has been much higher than expected, once entered into active study the drop-out rate has been moderate. Accession of new patients will be stopped this year. This study is also in the process of adding certain new techniques for evaluation of physiologic factors affecting bone metabolism: intestinal absorption tests (^{47}Ca), blood levels of a variety of vitamin D metabolites and fluoride tolerance tests. In addition, in association with this project members of the Physics Department, Hadassah Medical Organization, are being supported in the development of a relatively new technique of bone densitometry, Compton scattering, which it is hoped may be applied to the spine, a most difficult location in which to make such measurements, yet the most critical for assessment of the status of osteoporosis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 43002-14 MD

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Structure, Secretion and Mechanism of Action of Parathyroid Hormone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, MDB, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
A. M. Spiegel, M.D., Senior Investigator, MDB, NIAMDD
E. M. Brown, M.D., Senior Clinical Associate, MDB, NIAMDD
D. G. Gardner, M.D., Clinical Associate, MDB, NIAMDD
R. W. Downs, Jr., M.D., Clinical Associate, MDB, NIAMDD
M. Attie, M.D., Clinical Associate, MDB, NIAMDD
M. F. Brennan, M.D., Surgery Branch, National Cancer Institute
J. T. Potts, Jr., M.D., Massachusetts General Hospital, Boston
H. T. Keutmann, M.D. "
M. Rosenblatt, M.D. "
R. Windeck, M.D., Visiting Scientist, MDB, NIAMDD

COOPERATING UNITS (if any)
Endocrine Unit, Massachusetts General Hospital

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.50	2.50	2.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Little is known about the nature of circulating parathyroid hormone in clinical disease. It is the purpose of this project to isolate parathyroid hormone, determine the structure of the polypeptide, elucidate the mechanism of action of the hormone, and to develop a clinically useful test for circulating parathyroid hormone. From these studies it is expected that one can understand the pathophysiology of certain metabolic diseases of bone and endocrine disturbances. The entire structures of bovine, porcine and human parathyroid hormone have been determined. Synthetic polypeptides representing the first 34 residues of bovine and human parathyroid hormone have been prepared. These molecules show all the biological properties of the native hormonal polypeptide. A highly sensitive radioimmunoassay for the hormone has been developed and is being modified further for potential use as a specific clinical tool. Studies show that the mechanism of action of the hormone is mediated through direct hormonal activation of adenylate cyclase in bone and kidney. An isolated parathyroid cell system has been developed that allows studies of secretagogues for parathyroid hormone.

340-6040
(Rev. 10-76)

Objectives: To prepare purified parathyroid hormone in pilot-plant quantities; to study chemical properties and structure of the hormone; to relate chemical properties and structure to biological activity and to study the mechanism of action of the hormone; to develop a clinically useful test for circulating parathyroid hormone; to study the physiological regulation of secretion of the hormone.

Methods Employed: Pure parathyroid hormone is prepared from phenol extracts by methods previously described from this laboratory. The hormone is assayed in vivo in rats, in vitro by measuring hormonal activation of adenylate cyclase, or by a highly sensitive radioimmunoassay. Adenylate cyclase is measured by determining conversion of α-^{32}P-labeled ATP to cyclic 3',5'-AMP. Endogenous concentration of cyclic 3',5'-AMP or cyclic 3',5'-GMP is determined by radioimmunoassay.

Major findings: mechanism of action. Our earlier studies showed that the parathyroid status of animals is an important factor regulating excretion of cyclic AMP in the urine. This finding led to development of the thesis that cyclic AMP is involved in the mediation of parathyroid hormone action. Subsequent work showed that parathyroid hormone in vitro activates adenylate cyclase in the kidney as well as in bone, the two organs known to be physiological receptors for action of the hormone. Adenylate cyclase in the kidney is found in two different anatomic zones; adenylate cyclase in the cortex responds specifically to parathyroid hormone whereas that in the medulla responds to vasopressin. This work and numerous corroborative studies in other laboratories established that the receptor for parathyroid hormone in kidney resides within the renal cortical tubule as had been suspected but not proven from indirect in vivo physiological experiments.

Human parathyroid hormone. Parathyroid hormone has been purified from human parathyroid adenomas. Earlier studies proved that the human hormone is different immunologically from the bovine hormone. Amino acid analyses of the hormone purified from each source indicated approximately eight amino acid substitutions in the human hormone. The amino acid sequence has been completed for the first 37 amino acids at the amino-terminal end of the molecule (Drs. Potts and Rosenblatt). Based on this analysis, Drs. Potts and Rosenblatt (Boston) synthesized the tetratriacontapeptide representing the amino terminus of the molecule. The product is fully active in vitro and in vivo and studies have already been reported on clinical use of the peptide.
[Dr. G.D. Aurbach; Drs. Potts, Rosenblatt and Keutmann]

The entire amino acid sequence of human parathyroid hormone has now been completed (Keutmann, Sauer, Boehnert, Hendy and O'Riordan, Program of the 60th Meeting of The Endocrine Society, Abstract #477).

Parathyroid cells in vitro. Studies have continued with dispersed bovine and human parathyroid cells prepared by digestion with collagenase and DNase. Secretion of PTH from bovine cells is stimulated by β-adrenergic catecholamines, dopamine, PGE_2, secretin and cholera toxin, while it is inhibited by divalent cations, α-adrenergic catecholamines, $PGF_{2\alpha}$ and nitroprusside. There is a log-linear relationship for agonists between the increment in cAMP accumulation and the increase in rate of PTH release. The decrease in secretion rate with α-adrenergic agents and $PGF_{2\alpha}$ appears to be explainable by the reduction in cAMP levels effected by these agents. Increased calcium concentrations also lower cAMP, but this divalent cation also appears to have inhibitory effects independent of changes in intracellular cAMP. Cholera toxin increases both cAMP accumulation and secretion following a 30-60 minute lag analogous to that observed with other systems. In addition, it renders the cell more sensitive to the effects of other secretagogues on both cAMP accumulation and PTH release. It does not, however, alter the response to inhibitors of secretion.

Studies employing dispersed cells from pathologic human parathyroid glands suggest a variety of abnormalities in function. Secretion from 85% of glands of patients with primary hyperplasia were suppressed more than 50% by elevated calcium concentrations; in most of these cases, the "set point" for calcium (the concentration half-maximally inhibiting PTH release) was nearly normal (1.0-1.1 mM vs 1.0 for normal glands). In 38% of adenomas, on the other hand, PTH release was inhibited less than 50%. In the remainder, secretion was inhibited by higher than normal calcium concentrations (1.22-1.5 mM). In the two cases of parathyroid carcinoma, PTH release was inhibited less than 15% by elevated calcium concentrations.

Pathologic human tissue also differed from bovine tissue in response to secretagogues. In some cases, human tissue showed a β_1-response of cAMP accumulation to β-adrenergic agonists, while in other cases a β_2-response was observed. No responses to dopamine or secretin were observed, while histamine and glucagon elicited substantial increases in cAMP content in most cases. No inhibitory effects of α-adrenergic agonists or $PGF_{2\alpha}$ were observed, while PGE generally caused larger responses than observed with bovine cells.

Studies have also been carried out comparing the effects of elevated calcium concentrations on parathyroid function in vivo and in vitro in individual patients with primary hyperparathyroidism. In vitro studies were carried out with dispersed parathyroid cells; in vivo assessment of parathyroid suppressibility was carried out by determining the effects of intravenous calcium infusion on the nephrogenous component of urinary cAMP. In individual patients there was a close correspondence between suppressibility in vivo and in vitro, independent of the absolute degree of inhibition of secretion. Similar studies have been carried out in patients bearing forearm parathyroid autografts. These results suggest that the function of dispersed parathyroid cells in vitro reflects with reasonable accuracy parathyroid function in vivo. Moreover, they confirm the distinct calcium sensitivity of parathyroid adenoma vs. primary parathyroid hyperplasia.

These studies indicate the ability of dispersed parathyroid cells for investigating normal or abnormal parathyroid physiology. The mechanisms of secretagogue- and calcium-regulated PTH release may be studied in terms of interaction with specific receptors, effects on intracellular mediators and the exocytotic mechanism for secretion.
[Drs. Brown, Gardner and Brennan]

Studies using hypotonic lysates of dispersed bovine parathyroid cells have shown that dopamine can stimulate adenylate cyclase. The sensitivity to dopaminergic agonists and antagonists of the cyclase and intact cells (cyclic AMP accumulation and PTH release) were virtually identical suggesting that dopamine-stimulated PTH release is mediated through stimulation of adenylate cyclase. Further studies have shown the specificity of the receptor to be similar to other recently described dopamine-sensitive cyclases (D-1 receptors; D-2 receptors do not lead to stimulation of cyclase).
[Drs. Brown, Gardner and Attie]

Significance to Biomedical Research and Program of the Institute: Same as for July 1968-Setember 30, 1978.

Proposed Course of Project: Same as for July 1968-September 30, 1978.

Publications:

1. Aurbach, G. D., and Phang, J. M.: Vitamin D, parathyroid hormone, and calcitonin. In Mountcastle, V. B. (Ed.): Medical Physiology, 14th Edition. Saint Louis, The C. V. Mosby Co., 1978, in press.

2. Aurbach, G. D.: Parathyroid. In Beeson, P. S., McDermott, W., and Wyngaarden, J. B. (Eds.): Cecil-Loeb Textbook of Medicine, 15th Edition. Philadelphia, W. B. Saunders Company, 1979, pp. 2213-2224.

3. Gardner, D. G., Brown, E. M., Windeck, R., and Aurbach, G. D.: Prostaglandin E_2 stimulation of adenosine 3',5'-monophosphate accumulation and parathyroid hormone release in dispersed bovine parathyroid cells. Endocrinology 103: 577-582, 1978.

4. Brown, E. M., Hurwitz, S. H., and Aurbach, G. D.: Alpha-adrenergic inhibition of 3',5'-adenosine monophosphate accumulation and parathyroid hormone release from dispersed bovine parathyroid cells. Endocrinology 103: 893-899, 1978.

5. Brown, E. M., Gardner, D. G., Windeck, R. A., and Aurbach, G. D.: Relationship of intracellular cAMP accumulation to parathyroid hormone release from dispersed bovine parathyroid cells. Endocrinology 103: 2323-2333, 1978.

6. Windeck, R., Brown, E. M., Gardner, D. G., and Aurbach, G. D.: Effect of gastrointestinal hormones on isolated bovine parathyroid cells. Endocrinology 103: 2020-2026, 1978.

7. Brown, E. M., Gardner, D. G., Windeck, R. A., and Aurbach, G. D.: Cholera toxin stimulates 3',5'-adenosine monophosphate accumulation and parathyroid hormone release from dispersed bovine parathyroid cells. Endocrinology 104: 218-225, 1979.

8. Gardner, D. G., Brown, E. M., Windeck, R., and Aurbach, G. D.: Prostaglandin $F_{2\alpha}$ inhibits 3',5'-adenosine monophosphate accumulation and parathyroid hormone release from dispersed bovine parathyroid cells. Endocrinology 104: 1-7, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 43003-14 MD

PERIOD COVERED

October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on the Mode of Action of Thyrocalcitonin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.50	.25	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose is to study the interaction of calcitonin with its specific receptor target organs. The current investigations should provide further insight into the structure function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful for characterizing hormone receptors in kidney, bone and other tissues. Studies are in progress to characterize further the interaction of calcitonin with tissue receptors. It also will be of interest to solubilize the receptors and characterize them chemically.

PHS-6040
(Rev. 10-76)

Objectives: To study the interaction of calcitonin with its specific receptor target organs.

Methods Employed: The interaction of calcitonin with its receptors has been evaluated by determining adenylate cyclase as well as binding of radioiodinated calcitonin to membranes isolated from renal tissue and to lymphoid cells isolated from human and other species or grown in tissue culture.

Major Findings: A purification procedure for kidney membranes has been developed. It has been shown that the calcitonin receptor from the kidney is concentrated at the corticomedullary junction. The membranes which bind calcitonin and contain calcitonin-sensitive adenylate cyclase show low specific activities for renal brush border enzymes, indicating that calcitonin receptors are localized at an anti-luminal aspect of renal cells or at a site that does not contain brush borders. Utilizing a series of calcitonin analogs, dose response curves were compared for binding to receptors from kidney and bone. The dose response curves in the two tissues are virtually superimposable, suggesting that the calcitonin receptor in the two organs has a similar molecular structure. The interaction of the hormone-receptor complex with the adenylate cyclase enzyme is modified by the presence of guanine nucleotides. In the presence of GMPPNP the threshold for hormonal activation is lowered.

The relative renal responses to calcitonin analogs are similar whether determined by competitive binding or adenylate cyclase activation. Thus potency differences of the analogs tested are largely dependent upon effectiveness at the receptor level. Activity of the adenylate cyclase enzyme of human lymphocytes was not modulated by ambient or bound calcitonin.

Calcitonin receptors were identified on cultured human lymphocytes. The characteristics of these receptors are similar to those on rat renal and calvarial membranes as determined by competitive binding studies. The lymphocyte receptors, however, do not appear capable of modulating adenylate cyclase enzyme in that cyclic AMP was not detected after adding calcitonin to intact cells. Lymphocyte preparations should prove useful, however, in that they represent a readily obtainable human tissue containing calcitonin receptors. Results with this human material indicated that, although calcitonin polypeptides have undergone remarkable evolutionary changes in structure and effectiveness, there has been no significant corresponding change in biological nature of calcitonin receptors.

Significance to Biomedical Research and Program of the Institute: The current investigations should provide further insight into the structure-function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful characterizing hormone receptors present in kidney, bone, and other tissues.

Proposed Course of Project: Further studies are in progress to characterize further the interaction of calcitonin with its tissue receptors. It also will be of interest to solubilize calcitonin receptors and further characterize them. Purified lymphoid cell types are being characterized with respect to the density of calcitonin receptors on their surface. The whole cell system also may be applied to evaluate the regulation of receptor metabolism in vitro and in human disease states.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 43004-14 MD

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on pseudohypoparathyroidism and related disorders

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB, NIAMDD
A. M. Spiegel, M.D., Senior Investigator, MDB, NIAMDD
E. M. Brown, M.D., Senior Clinical Associate, MDB, NIAMDD
D. G. Gardner, M.D., Clinical Associate, MDB, NIAMDD
R. W. Downs, Jr., M.D., Clinical Associate, MDB, NIAMDD
M. Attie, M.D., Clinical Associate, MDB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.75	.50	.25

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. Our findings that parathyroid hormone action is likely mediated through activation of adenylate cyclase, led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3',5'-AMP in the urine. In normal subjects as well as usual forms of hypoparathyroidism, parathyroid hormone causes a 10- to 60-fold increase in urinary cyclic AMP (cAMP). This response is abnormal (slight or no increased cAMP excretion) in pseudohypoparathyroidism, indicating that there is a defective parathyroid hormone-adenylate cyclase complex in the kidney in this disorder.

240-6040
(Rev. 10-76)

Project Description. In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. Albright proposed that this disorder was attributable to lack of end organ sensitivity to normally-secreted endogenous parathyroid hormone. Our findings that parathyroid hormone action is likely mediated through activation of adenylate cyclase, led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3',5'-AMP in the urine. Pseudopseudo-hypoparathyroidism is a variant of the disorder and may occur in the same families with pseudohypoparathyroidism. Pseudopseudohypoparathyroidism is characterized by similar constitutional features but no clinical evidence of hypoparathyroidism. Several other syndromes including Gardner's syndrome, basal cell nevus syndrome, syndrome of calcification of basal ganglia, and vitamin D-resistant osteomalacia have also been reported in the past as resistant to the phosphaturic action of parathyroid hormone. Current studies have been extended to include testing patients in these categories as well.

Methods: Parathyroid hormone purified in our laboratories was sterile-filtered for clinical use through courtesy of the N.I.H. Pharmacy. Radio-immunoassay of parathyroid hormone was carried out as described previously. Measurement of 3',5'-AMP was performed according to the procedure devised in this laboratory.

Major Findings: In normal subjects, parathyroid hormone causes a 10- to 60-fold rise in urinary excretion of cyclic AMP within 30 minutes or less after intravenous administration of the hormone. A similar response has been observed in idiopathic, hypoparathyroid, surgical hypoparathyroid, and pseudopseudohypoparathyroid subjects (the latter category show the constitutional changes but do not have laboratory evidence of hypoparathyroid-ism). Parathyroid hormone caused little or no increase in excretion of cyclic AMP in patients with pseudohypoparathyroidism. In one case, studied through the cooperation of a clinical investigator at another hospital, there was a moderate rise in excretion of cyclic AMP; however, the diagnosis in this instance may be incorrect, and as yet we have not had the opportunity of seeing the patient at the Clinical Center. Patients with this disorder who fail to respond to parathyroid hormone showed a similarly defective response after prolonged calcium infusion. Parathyroid hormone was detectable in the plasma of pseudohypoparathyroid subjects before infusion of calcium but not after. This experiment tended to rule out the possibility that an abnormal endogenously secreted hormone immunologically reactive but biologically inert interfered with the action of exogenous parathyroid hormone.

The best evidence to date is that pseudohypoparathyroidism represents a genetic defect inherited as a sex-linked, dominant trait. There have been no documented cases of direct male-to-male transmission of the disease

and the sex incidence is approximately 2:1 female-to-male. Several pedigrees have been described wherein pseudophyoparathyroid subjects were progeny of pseudopseudohypoparathyroid parents or vice versa. One family studied at the N.I.H. included a mother with pseudopseudohypoparathyroidism and her daughter with pseudohypoparathyroidism. The daughter failed to respond to exogenous hormone but the mother responded normally. We have also observed that, although the response to pseudopseudohypoparathyroidism is normal to exogenous hormone, there is a significantly increased rate of baseline excretion of cyclic AMP in this group. This observation may be of importance in further delineation of the precise genetic mechanism involved in transmission of the disorder. It appears likely that the cause of the disorder may be the existence of genetically deficient or defective adenylate cyclase in the bone and kidney of these subjects.

There is an association of hypothyroidism with pseudohypoparathyroidism and in a few such cases the hypothyroidism has been attributed to selective deficiency of thyrotropin. It seems possible that hypothyroidism in pseudohypoparathyroidism might reflect an abnormal receptor adenylate cyclase complex in the central nervous system or anterior pituitary analogous to the defect in the receptor for parathyroid hormone in kidney and bone. Three patients with coexistent hypothyroidism and pseudohypoparathyroidism were given thyrotropin-releasing hormone as part of a study designed to localize the site of the thyroid defect. One case showed an abnormally low basal concentration of thyrotropin in plasma, but gave a normal response to thyrotropin-releasing hormone. In the two other cases, a mother and daughter, there were high basal concentrations of thyrotropin in plasma. The mother was tested with TRH and showed the exaggerated response to TSH secretion characteristic of primary hypothyroidism. The defect in the first case appears to be at the level of the hypothalamus or higher. The defect in the second two cases may represent an abnormality of the thyroid receptor for TSH itself.

Patients with Cushing's syndrome, basal cell nevous syndrome, syndrome of calcification of the basal ganglia, and vitamin D-resistant osteomalacia showed normal responses to parathyroid hormone as determined by urinary excretion of 3',5'-AMP. It was concluded that the classical phosphaturic response to parathyroid hormone is neither precise nor sensitive enough to discriminate normal subjects from those refractory to parathyroid hormone.

Kidney tissue was obtained from a patient with pseudohypoparathyroidism who died suddenly with complications of a massive pulmonary embolus. It was demonstrated in vitro that the membrane fraction of this kidney contained adenylate cyclase activity which was stimulatable by parathyroid hormone. Thus, it was concluded that pseudohypoparathyroidism did not represent a total lack of the renal parathyroid hormone-sensitive adenylate cyclase system.

The role of $1,25\text{-}(OH)_2\text{-}D_3$, the most potent natural metabolite of cholecalciferol, has been assessed in hypocalcemic states. We have discovered a family with a unique disturbance of calciferol metabolism. Two siblings have

rickets and osteomalacia attributable to decreased target tissue sensitivity to 1,25-$(OH)_2$-D. When their serum concentrations of 1,25-$(OH)_2$-D are maintained by various calciferol analogs at concentrations approximately 20-fold normal, their disorder can be fully controlled.

Significance to Biomedical Research and Program of the Institute: Same as reported for July 1, 1968-September 30, 1978.

Proposed Course of Project: Same as reported for July 1, 1968-September 30, 1978.

Publications:

Marx, S. J., Spiegel, A. M., Brown, E. M., Gardner, D. G., Downs, R. W., Jr., Attie, M., Hamstra, A. J., and DeLuca, H. F.: A familial syndrome of decrease in sensitivity to 1,25-dihydroxyvitamin D. J. Clin. Endocinol. Metab. 47: 1303-1310, 1978.

White, B. J., and Marx, S. J.: Dermatoglyphic and radiographic findings in a mother and daughter with pseudohypoparathyroidism. Clinical Genetics 13: 359-368, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER A01 AM 43005-14 MD

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Hormone Receptors, Adenylate Cyclase and Cyclic AMP-Mediated Ion Transport

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
A. M. Spiegel, M.D., Senior Investigator, MDB, NIAMDD
E. M. Brown, M.D., Senior Clinical Associate, MDB, NIAMDD
D. G. Gardner, M.D., Clinical Associate, MDB, NIAMDD
R. W. Downs, Jr., M.D., Clinical Associate, MDB, NIAMDD
M. Attie, M.D., Clinical Associate, MDB, NIAMDD
J. D. Gardner, M.D., Chief, Digestive Diseases Branch, NIAMDD
T. Nielsen, M.D., Laboratory of Nutrition and Endocrinology, NIAMDD
M. Rodbell, M.D., LNE, NIAMDD

COOPERATING UNITS (if any)
Section on Gastroenterology, Digestive Diseases Branch, NIAMDD
Laboratory of Nutrition and Endocrinology, NIAMDD

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.50	2.50	2.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To isolate and purify specific hormone receptors in particular receptor tissues; to map function of the receptor against activation of adenylate cyclase; to determine the nature of the link between generation of cyclic 3',5'-AMP and solute transport in particular cell systems. β-adrenergic receptors have been identified using radioiodinated hydroxybenzylpindolol (1-(1-para-hydroxyphenyl-2-methyl-propylamino)-3-(4-indolyloxy)-2-propanol), a high-affinity β-blocker. This compound binds specifically to β-receptor sites on cell membranes. Kinetic and equilibrium studies identified a single class of high affinity ($2\text{-}4 \times 10^{10} M^{-1}$) receptor sites on turkey erythrocyte membranes, 200-400 sites per cell. A series of β-adrenergic agonists and inhibitors compete for this binding site with affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. The stereospecificity and the correspondence between affinity for site and biological effectiveness indicate that binding of iodohydroxybenzylpindolol validly reflects interaction with β-adrenergic receptors in several cell systems.

HHS-6040
(Rev. 10-76)

Objectives: To isolate and purify specific hormone receptors in particular receptor tissues; to map function of the receptor against activation of adenylate cyclase; to determine the nature of the link between generation of cyclic 3',5'-AMP and solute transport in particular cell systems.

Methods Employed: Polypeptide and catecholamine hormones are purified and iodinated by standard methods or methods developed in the laboratory specifically for that purpose. An affinity resin bearing GTP linked through the γ-phosphate was synthesized and utilized in isolation of the guanine nucleotide regulatory protein controlling adenylate cyclase activity. Rate of association of hormones is determined by incubating plasma membrane fractions or whole cell with labeled hormones. The kinetics of binding is determined by rate measurements as well as assessment of steady state conditions. Results are analyzed by double reciprocal, Scatchard, or four-parameter logistic (Rodbard) models utilizing computer programs based on these models. Specific hormone binding is defined in terms of dissociation constant (K_m) of the system. Physiologically significant binding shows K_m's compatible with the dose-response relationship recognized for the hormone at the physiological level.

The guanine nucleotide regulatory unit has been isolated from the turkey erythrocyte adenylate cyclase complex with GTP affinity chromatography. Treatment of turkey erythrocyte membranes with β-adrenergic agonist (isoproterenol) and GMP permits solubilization of the enzyme in a guanine nucleotide-responsive state. Incubation of the soluble enzyme preparation with GTP (linked via the γ-phosphate) agarose results in substantial (>75%) depletion of Gpp(NH)p-responsive enzyme activity from the supernatant fraction. Biospecific elution of the affinity matrix (buffer containing 0.2 mM Gpp(NH)p) yields a fraction with negligible intrinsic adenylate cyclase activty but capable of restoring at least 50% of the Gpp(NH)p-responsive activity to the depleted supernatant fraction. These results suggest that the guanine nucleotide regulatory unit is reversibly associated with the catalytic unit of the solubilized holoenzyme. Using GTP affinity chromatography, the guanine nucleotide regulatory unit may be purified 50-100 fold over the detergent extract of turkey erythrocyte membranes. We have also shown that the affinity-purified guanine nucleoitde regulatory unit from turkey erythrocytes restores both guanine nucleotide- and fluoride-responsive adenylate cyclase activity to a mutant mouse lymphoma cell line ($S49\ CYC^-$). [In collaboration with T. Nielsen and M. Rodbell, LNE, NIAMDD] These observations demonstrate that heterologous guanine nucleotide regulatory units and catalytic units are capable of functional interaction and suggest that the structure of the guanine nucleotide regulatory unit is evolutionarily conserved. Furthermore, the ability of affinity-purified guanine nucleotide regulatory unit to restore fluoride-responsive activity is a direct demonstration that the guanine nucleotide regulatory unit is involved in the mechanism of fluoride action.

The guanine nucleotide regulatory unit is necessary for fluoride activation of adenylate cyclase, and changes in the nature of the guanine nucleotides bound to the regulatory unit influence fluoride-stimulated adenylate cyclase

activity. Incubation of membranes with isoproterenol and GMP causes a decline in fluoride-stimulated enzyme activity which can be reversed by GDP or GTP. A GDP analog, guanosine-α,β-methylene diphosphonate, can also restore enzyme activity under conditions where conversion to its form ($GP(CH_2)PP$) does not occur. Direct analysis of the guanine nucleotide bound at isoproterenol-dependent regulatory sites, using the method of Cassel and Selinger (PNAS 75:4155-4159, 1978), shows that fluoride does not enhance conversion of GDP to GTP, nor does fluoride inhibit the hydrolysis of GTP to GDP at regulatory sites. Additional studies have been performed with another analog of GDP, guanosine 5'-diphosphate-β-sulfur (GDP-β-S), which interacts with the guanine nucleotide regulatory unit in a hormone-dependent fashion. When GDP-β-S is bound to the regulatory unit, fluoride stimulation of adenylate cyclase does not occur, even with excess GTP in the incubation medium. Conversely, when GDP is bound to the regulatory unit, GDP-β-S does not inhibit fluoride activation of adenylate cyclase, indicating that exchange of the guanine nucleotide bound at the regulatory site with nucleotides in the medium is not necessary for enzyme activation by fluoride. Endogenous GDP, tightly bound to the guanine nucleotide regulatory unit, is sufficient for supporting fluoride stimulation of adenylate cyclase activity.

Significance to Biomedical Research and Program of the Institute: Human erythrocyte, devoid of adenylate cyclase activity, contains a guanine nucleotide regulatory unit. This unit can be quantitated in various disease states, e.g. pseudohypoparathyroidism, and serve as a probe of one link in chain of events between hormone binding to cell surface and physiologic effect.

Proposed Course of Project: Further purification of the guanine nucleotide regulatory unit and study of its structure and function.

Publications:

1. Aurbach, G. D., and Brown, E. M.,: Hormone Receptors, Cyclic Nucleotides, and Control of Cell Function. In Freinkel, N. (Ed.): Contemporary Metabolism, Vol. I. New York, Plenum Publishing Corporation, 1979, pp. 193-233.

2. Brown, E. M., Spiegel, A. M., Gardner, J. D., and Aurbach, G. D.: Direct identification and characterization of beta-adrenergic receptors and functional relationship of adenylate cyclase. In Birnbaumer, L. and O'Malley, B. W. (Eds.): Receptors and Hormone Action, Vol. III. New York, Academic Press, 1978, pp. 101-131.

3. Downs, R. W., Jr., Spiegel, A. M., Singer, M., and Aurbach, G. D.: Fluoride stimulation of adenylate cyclase is guanine nucleotide dependent. J. Biol. Chem., in press.

4. Spiegel, A. M., Downs, R. W., Jr., and Aurbach, G. D.: Separation of a guanine nucleotide regulatory unit from the adenylate cyclase complex with GTP affinity chromatography. J. Cyclic Nucleotide Res. 5: 3-17, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER A01 AM 43006-04 MD

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Study of Hyperparathyroidism: Etiology, diagnosis and treatment.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

G. D. Aurbach, M.D., Chief, Metabolic Diseases Branch, NIAMDD
S. J. Marx, M.D., Senior Investigator, MDB,NIAMDD
A. M. Spiegel, M.D., Senior Investigator, MDB, NIAMDD
E. M. Brown, M.D., Senior Clinical Associate, MDB, NIAMDD
D. G. Gardner, M.D., Clinical Associate, MDB, NIAMDD
R. W. Downs, Jr., M.D., Clinical Associate, MDB, NIAMDD
M. Attie, M.D., Clinical Associate, MDB, NIAMDD
J. L. Doppman, M.D., Radiology Department, Clinical Center
M. Brennan, M.D., Surgery Branch, NCI
H. DeLuca, M.D., Department of Biochemistry, Univ. of Wisconsin
J. T. Potts, Jr., M.D., Endocrine Unit, Massachusetts General Hospital
J. D. Gardner, M.D., Digestive Diseases Branch, NIAMDD

COOPERATING UNITS (if any)
Radiology Department, CC; Surgery Branch, NCI; Department of Biochemistry, University of Wisconsin; Endocrine Unit, MGH; Digestive Diseases Branch, NIAMDD

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.50	2.25	1.25

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project goal is the evaluation and treatment of hyperparathyroidism. Patients with persistent postoperative hyperparathyroidism are referred for evaluation and treatment. Hereditary hyperparathyroidism in particular is under investigation in the hopes of delineating hereditary molecular abnormalities in glandular regulation, as exemplified in the multiple endocrine neoplasia syndromes. Evaluation ranges from epidemiologic studies of families to in-house studies of patients and to in vitro analyses of excised tissue. Techniques currently being employed and improved include radioimmunoassay of parathyroid hormone, selective arteriography and selective venous sampling for parathyroid hormone, parathyroid gland cryopreservation and autotransplantation, and transcatheter parathyroid gland infarction.

PHS-6040
(Rev. 10-76)

Objectives: To study the regulation of parathyroid gland function. To develop and evaluate diagnostic tests of parathyroid function. To develop new methods for localization of hyperfunctioning parathyroid tissue. To evaluate methods of treatment of hyperparathyroidism.

Methods employed: Patients with suspected hyperparathyroidism are referred for evaluation and treatment. Evaluation includes radioimmunoassay of circulating parathyroid hormone, and of plasma and urinary cyclic AMP. Where appropriate, preoperative evaluation includes selective arteriography and venography with selective sampling for PTH determination and computerized tomography of neck and mediastinum. In selected cases adenomatous parathyroid glands are ablated by transarterial infusion of various agents. Parathyroid exploration is done by the Surgery Branch, NCI. If postoperative hypoparathyroidism is considered highly likely, parathyroid tissue may be simultaneously autotransplanted to a forearm muscle. Excised parathyroid tissue either fresh or cryopreserved is evaluated in vitro after preparation of dispersed cells. If postoperative hypoparathyroidism is considered possible, tissue is cryopreserved for subsequent autotransplantation. Techniques for cryopreserving and grafting parathyroid tissue are being studied in an experimental model with adult dogs.

Major findings: Diagnosis and clinical findings: A large number of patients with primary hyperparathyroidism have been treated and their clinical and chemical findings have been described. Weakness was noted more commonly than had previously been appreciated and muscle biopsy showed atrophy of type I and especially type II fibers in a manner diagnostic of denervating disease. Studies of neuromuscular function in subjects with secondary hyperparathyroidism showed similar clinical and histologic findings. The most useful diagnostic tests for hyperparathyroidism have been hypercalcemia associated with nonsuppressed or increased parathyroid hormone concentrations in peripheral blood and increased urinary excretion of cyclic AMP. These findings revert rapidly to normal following successful parathyroidectomy. Intestinal absorption of calcium is increased preoperatively and undoubtedly has a central role in causing hypercalcemia.
[G.D.Aurbach, S.J.Marx, A.M.Spiegel, E.M.Brown, D.G.Gardner, R.W. Downs, Jr., and M. Attie, MDB, NIAMDD; M. Brennan, Surgery Branch, NCI]

Several studies have utilized urinary cyclic AMP excretion as a parameter of parathyroid hormone action:

Measurement of intraoperative urinary cAMP excretion in patients undergoing neck exploration for primary hyperparathyroidism has shown that successful removal of abnormal parathyroid tissue is followed by a rapid decline to low levels (<1.5 μmol/g creatinine). In patients in whom surgery was not curative, levels remained elevated. These results suggest that monitoring UcAMP excretion intraoperatively (utilizing techniques for rapid radioimmunoassay of cAMP) could aid in operative management of patients with primary hyperparathyroidism.

Lithium has several actions (e.g. inhibition of antidiuretic hormone action) that have led to the hypothesis that it is a general inhibitor of adenylate cyclase and may be useful in treating diseases characterized by excessive hormone activation of this enzyme. The effects of lithium on PTH activation of renal adenylate cyclase were studied in patients receiving lithium for treatment of manic depressive disorders and in patients with primary hyperparathyroidism. We found no inhibition by lithium of the rise in UcAMP excretion following PTH infusion and no effect of lithium on serum calcium or tubular reabsorption of phosphate in patients with primary hyperparathyroidism. Lithium appears to be unable to inhibit PTH activated adenylate cyclase at doses that can be achieved clinically.

Familial hyperparathyroidism. Surveys of families of patients with primary parathyroid hyperplasia show that hereditary disease is very common (approximately 50% in our series of 25 index cases with primary parathyroid hyperplasia). In two large families a novel form of hyperparathyroidism was found wherein hypercalcemia occurs without hypercalciuria. Unlike typical cases of familial hyperparathyroidism, the penetrance of hypercalcemia approached 100% in the first two decades with severe expression even in one neonate. Review of results of surgery at the N.I.H. and elsewhere has shown that all of 6 patients in this category treated by subtotal parathyroidectomy had persistent postoperative hypercalcemia. While elevated concentrations of glucagon and/or gastrin were common in subjects with familial multiple endocrine neoplasia type I, these were not elevated in hypercalcemic members of these two families. In these families renal clearances of filtered calcium and magnesium are both lower than in typical primary hyperparathyroidism and circulating parathyroid hormone levels are lower than in typical primary hyperparathyroidism.
[S.J.Marx, A.M.Spiegel, E.M.Brown, D.G.Gardner, R.W.Downs,Jr., M.Attie, J.L.Stock, and G.D.Aurbach, MDB,NIAMDD; J.Gardner, DDB,NIAMDD]

Application of new radiologic techniques: A series of studies has been carried out utilizing selective venous catheterization and a radioimmunoassay for parathyroid hormone for localization of parathyroid adenomas. A high degree of success has been obtained in localizing these tumors preoperatively. The catheter is passed through the femoral vein and thence through the major veins to reach the neck where selective samples are taken from each inferior and superior thyroid vein. Radioimmunoassays show that veins draining parathyroid adenomas frequently contain concentrations of hormone 10- to 20-fold or more greater than the concentration in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in one vein draining the adenoma. The contralateral vein shows concentrations equal to samples from the general circulation. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on each side greater than concentrations in peripheral plasma. Usually in these cases the concentration ratio is not as high as for single adenomas. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by nonparathyroid cancers.

Thyroid and internal mammary arteriography enables preoperative identification of hyperfunctioning parathyroid tissue in 30% or more of cases and is also helpful in defining venous drainage to facilitate selective catheterization. In eight cases of mediastinal parathyroid adenoma treatment was accomplished by transarterial infusion of hypertonic glucose, gelfoam or autologous clot to occlude the catheterized parathyroid artery. Seven appear fully cured and one partially. There was no significant morbidity to these patients who would otherwise have required sternotomy. [G.D.Aurbach, S.J.Marx, A.M.Spiegel, E.M.Brown, D.G.Gardner, R.W. Downs, Jr., M. Attie, and J.L.Stock, MDB,NIAMDD; J.Doppman, Radiology Dept., C.C., NIH]

Autotransplantation and cryopreservation of parathyroid: Persistent postoperative hyperparathyroidism or hypoparathyroidism are potential problems in patients with primary parathyroid hyperplasia or previous unsuccessful parathyroid exploration. These problems may be lessened if parathyroid tissue can be cryopreserved. We have developed experience with cryopreservation and autotransplantation of parathyroid glands in normal dogs and also in a few patients who developed permanent hypoparathyroidism postoperatively. Avoidance of tissue trauma or rapid changes in temperature or pH was essential for tissue preservation. Canine parathyroid tissue has been successfully autotransplanted after cryopreservation for as long as nine months and function has been documented by measuring release of immunoreactive PTH and by induction of hypocalcemia following graft excision. Function of autografted parathyroid tissue in vivo correlates well with assessment in vitro. One patient who received an autograft from a parathyroid adenoma developed rapid recurrence of primary hyperparathyroidism. Subsequently his tissue showed highly abnormal function in vitro and in vivo. Graft excision was required and he became hyoparathyroid. [G.D.Aurbach, S.J.Marx, A.M.Spiegel, E.M.Brown, D.G.Gardner, R.W.Downs,Jr., M.Attie, and J.L.Stock, MDB,NIAMDD; M. Brennan, Surgery Branch, NCI]

Significance to Biomedical Research and Program of the Institute: As an outgrowth of these studies, methods of localizing and treating hypersecreting tissues of several types are being improved. Hyperparathyroidism is a prototype of endocrine hyperfunction. Some patients have hyperparathyroidism in combination with hereditary hyperfunction of other endocrine glands including pituitary and pancreas. These hyperfunctions are usually benign but occasionally become cancerous. By studying families with hereditary disease we hope to gain insight into specific molecular lesions that cause loss of normal cellular regulation in these tissues. This collaborative program offers comprehensive treatment for a select group of patients who have proven difficult or impossible to treat in other centers in the United States and abroad.

Proposed Course of Project: Patients with suspected primary hyperparathyroidism will be accepted for evaluation and treatment. We will continue to study familial patterns of hyperparathyroidism and efforts will continue towards improving techniques for assessment of parathyroid hormone by radioimmunoassay or bioassay. We will continue to explore new methods of localizing

hyperfunctioning parathyroid tissue. The applicability of parathyroid embolization and the role of parathyroid autotransplantation will be investigated further. Patients in the entire program will provide a source of abnormal parathyroid glands to be studied in detail in vitro. Patients with parathyroid transplants will be ideal test subjects for putative parathyroid secretagogues.

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Annual Report of the Digestive Diseases Branch

SUMMARY OF BRANCH ACTIVITIES

The Digestive Diseases Branch has two sections (Section on Gastroenterology and Section on Diseases of the Liver). The Section on Diseases of the Liver has two senior physicians and 3 clinical associates; the Section on Gastroenterology has three senior physicians and 4 clinical associates.

Detailed summaries of the activities of each section precedes the individual project reports. Both sections are engaged in investigations of basic biologic processes (e.g. hormone action, membrane transport, cellular and humoral immunology) and are attempting to apply this information to understand the pathophysiology of various disorders involving the liver and gastrointestinal tract. Both sections are also involved in attempts to improve therapy of clinical disorders such as neoplasms associated with overproduction of gastrointestinal hormones, and fulminant hepatic failure.

Section on Gastroenterology

During the past year we have extended our studies of the biochemical basis of action of gastrointestinal hormones on their target tissues and have centered our investigations of patients with clinical syndromes caused by tumor production of one or more gastrointestinal hormones.

We have developed homogenous suspensions of viable, hormone-responsive cells from various gastrointestinal tissues e.g. gastric parietal cells, pancreatic acinar cells, small intestinal epithelial cells, colonic epithelial cells, colonic mucus-producing cells, and gastric smooth muscle cells. During the coming year we plan to develop homogenous suspensions of hepatocytes, hepatic sinusoidal lining cells and gastric mucus-producing cells. We plan to use these preparations to investigate various distinct cellular functions such as endocytosis of protein by hepatocytes, exocytosis of protein by pancreatic acinar cells, mucus synthesis and secretion by colonic mucus-producing cells, etc. We also plan to extend our present studies of the biochemical basis of action of various hormones, neurotransmitters and other chemical agents on these different cells.

In April, 1979 the Section on Gastroenterology conducted a workshop sponsored by the American Gastroenterological Association on techniques for preparating suspension of cells from various gastrointestinal tissues. This workshop was attended by approximately 50 active investigators and was sufficiently successful that we are considering repeating this workshop on an annual basis.

During the past year we have collaborated with several different peptide chemists and have succeeded in preparing radiolabeled peptides that enable us to identify each of the six different classes of hormone receptors on pancreatic acinar cells. During the coming year we plan to use these ligands to examine hormone receptors on other gastrointestinal tissues.

Serendipitously we discovered a competitive antagonist of the action of cholecystokinin. This antagonist, $N_6,O^{2'}$-dibutyryl cyclic GMP, appears to act

by competing with CCK for occupation of membrane receptors. During the coming year we plan to explore the structural specificity required for antagonist action and to search for endogenous antagonists of the action of cholecystokinin. Since cholecystokinin has been implicated as a regulator of food intake in animals and man, identification of an endogenous antagonist would be particularly helpful in furthering our understanding of the regulation of satiety.

We are currently treating approximately 30 patients with Zollinger-Ellison Syndrome with cimetidine. This drug appears to constitute effective therapy in this disorder and obviates the need for total gastrectomy. It has also become clear that most patients with Zollinger-Ellison syndrome will die from effects of the tumor per se not from effects of its secreted products. We plan to continue to explore various forms of antineoplastic therapy in patients with Zollinger-Ellison Syndrome.

A substantial majority of patients with the pancreatic cholera syndrome respond satisfactorily to streptozotocin. In those patients who do not respond, management of the voluminous diarrhea is a major clinical challenge. We are in the process of examining various pharmacologic agents for their abilities to ameliorate diarrhea in patients with pancreatic cholera syndrome.

Section on Diseases of the Liver

The Section on Diseases of the Liver is currently conducting six principal studies.

I. Studies of Cellular Immune Function in Chronic Hepatitis and Primary Biliary Cirrhosis

The role of abnormal immune mechanisms in the pathogenesis and perpetuation of the hepatobiliary lesions in chronic hepatitis and primary biliary cirrhosis (PBC) is being studied. We have previously shown that PBC is associated with unequivocal defects in (i) the spontaneous cytotoxic function of lymphocytes which lack surface immunoglobulin but bear Fc receptors and (ii) the C3b-receptor mediated clearance function of Kupffer cells. In addition, we have also shown that at least the majority of cells in peripheral blood which are responsible for spontaneous cell mediated cytotoxicity, mitogen-induced cellular cytotoxicity and antibody-dependent cellular cytotoxicity do not bear complement receptors.

Further studies have been designed to determine whether there is a defect in immune regulation in patients with PBC. T cell mediated help and suppression of pokeweed mitogen-induced immunoglobulin synthesis have been studied in vitro using radioimmunoassays to measure IgM and IgG. Cultures containing unirradiated T cells and B cells from normal individuals showed augmentation of immunoglobulin (Ig) synthesis at low T:B cell ratios (helper T cell effect) and inhibition of Ig synthesis at high T:B cell ratios (suppressor T cell effect). Cultures containing irradiated T cells from normal individuals showed augmentation of Ig synthesis at all T:B cell ratios studied, indicating marked reduction of suppressor T cell function by irradiation. In contrast, cultures containing unirradiated T cells and autologous B cells from patients with PBC did not show inhibition of Ig synthesis at high T:B cell ratios, but instead showed progressive increase in Ig synthesis with increasing T:B cell ratios up to the highest

T:B cell ratio studied. Ig synthesis by cultures containing irradiated T cells and autologous B cells from patients with PBC was similar to that by corresponding cultures of cells from normal subjects. These findings indicate that the diminished inhibition of Ig synthesis is attributable to a deficiency of suppression, rather than an excess of T cell help. Allogeneic mixtures of cells from normal individuals and patients with PBC showed that while B cells from patients with PBC synthesized normal amounts of Ig in the presence of normal T cells, T cells from patients with PBC caused marked enhancement of Ig synthesis by normal B cells. Thus, the abnormal regulation of Ig synthesis observed in PBC is most likely due to a deficiency of suppressor T cell function, rather than a B cell defect. Patients with other liver diseases characterized by chronic cholestasis, cirrhosis, or hepatocellular necrosis did not exibit an analogous abnormality of Ig synthesis in vitro to that found in PBC. Thus, we have shown that PBC is associated with a defect of immune regulation characterized by a diminished capacity of T cells to inhibit Ig synthesis in vitro.

To determine whether patients with PBC have abnormalities of T-cell recognition phenomena, autologous and allogeneic mixed lymphocyte reactions (MLR's) are being studied. These studies involve measuring the incorporation of ^{3}H-thymidine by T cells in response to autologous or allogeneic T-cell depleted peripheral blood mononuclear cells. Preliminary data in patients with PBC and other liver diseases and in normal control subjects suggest a significant reduction in the autologous MLR but a normal allogeneic MLR in PBC. Further experiments are in progress to investigate the role which serum antibodies and immune complexes may play in the mediation of this defect and to determine if there is a deficiency of the response of a particular subpopulation of T cells in the autologous MLR in PBC, such as suppressor T cells or cytotoxic effector cells. (E. A. Jones, S. P. James, C. O. Elson [NCI], W. Strober [NCI], J. H. Hoofnagle).

II. Studies on Chronic Hepatitis B Surface Antigen (HBsAg) Carriers

Studies have been initiated on the chronic HBsAg carrier state and chronic hepatitis associated with HBsAg. Seventeen patients have been enrolled in an extended study of the natural history of their chronic hepatitis and chronic infection with hepatitis B virus. Twelve patients have now been followed for at least six months and ten have had percutaneous liver biopsies. Assays for hepatitis B virus and hepatitis A virus markers have been established in the laboratory including HBsAg and titer, anti-HBs, anti-HBc, HBeAg, anti-HBe and anti-HAV. DNA-polymerase is also being assayed.

These studies are preliminary investigations aimed at defining a group of persons with stable chronic infections with hepatitis B virus who would be candidates for anti-viral therapy. At present the antiviral agent phosphonoformate is undergoing animal and in vitro studies for possible treatment of hepatitis B virus infection. If efficacy is seen in experimental animals and if toxicity data demonstrate that it is safe, this drug will be tested in the patients under study.

Various immunologic and virologic characteristics of HBsAg are under study. HBsAg is being purified from the plasma of chronic HBsAg carriers. The metabolic half-life and fate of HBsAg is under investigation using radiolabeled

HBsAg in experimental animals. Studies of the turnover of radiolabeled HBsAg will eventually be applied to chronic HBsAg carriers to define the synthetic and fractional catabolic rates of this antigen. The turnover data will be correlated with the titer of HBsAg and clinical activity of the associated liver disease.

HBsAg has recently been found in commercial preparations of immune serum globulin (ISG). However, this HBsAg is present as an immune complex with anti-HBs. The effect of ISG in ameliorating or preventing type B hepatitis may be due to the presence of this HBsAg-antibody complex. The differential immunogenicity of HBsAg alone versus HBsAg and antibody is being investigated in laboratory animals and the effect of anti-HBs on the metabolic fate of HBsAg is under study. (J. H. Hoofnagle, J. Gerin [Georgetown University], E. A. Jones, D. F. Schafer, S. P. James, Rodney Johnson [NIAID], Robert H. Purcell [NIAID], Stephen Feinstone [NIAID]).

III. Studies of the Properties of Desialylated Human Lymphocytes

We have previously shown that desialylated human lymphocytes are cytotoxic to hepatocytes and that this phenomenon is probably mediated by the interaction between desialylated glycoproteins on the surface of the lymphocytes and the hepatic binding protein on hepatocytes which specifically binds terminal galactosyl residues. It is known that infused desialylated lymphocytes are preferentially sequestered in the liver. However, after infusions of autologous desialylated lymphocytes into rabbits neither serial values for serum transaminases nor hepatic histology were indicative of the induction of hepatocellular injury. This negative finding suggested that the hepatic sequestration of desialylated lymphocytes *in vivo* might occur by a mechanism independent of their interaction with hepatic binding protein. Such a mechanism could involve the binding to these cells of the previously identified circulating natural antibody which specifically recognizes antigens on the surface of lymphocytes which are exposed by desialylation. Depletion of complement *in vivo* with cobra venom factor had no effect on the selective hepatic uptake of the label of ^{51}Cr-labeled desialylated peripheral blood mononuclear cells in the rabbit. However, the prior incubation of these cells with rabbit IgG Fab fragments reduced the hepatic uptake of label to values similar to those observed with ^{51}Cr-labeled intact peripheral blood mononuclear cells. These findings are compatible with the hypothesis that desialylated lymphocytes *in vivo* become coated with naturally occurring antibodies and that subsequent recognition of cell bound antibody by Fc receptors on Kupffer cells mediates their selective hepatic uptake (E. A. Jones, C. J. Steer, S. P. James).

IV. The Use of Artificial Hepatic Support Devices for the Treatment of Fulminant Hepatic Failure

We have developed a highly reproducible model of fulminant hepatic failure (FHF) in an inbred strain of rabbits using the selective hepatotoxin galactosamine hydrochloride. This model resembles human FHF in all important respects.

To investigate the efficacy of various hemoperfusion devices and schedules in the treatment of the model, a hepatic support system for the removal of potentially encephalopathogenic substances from the plasma of rabbits is being developed. In preliminary experiments in which the blood of normal rabbits was

perfused through hemoperfusion circuits which contained silicone rubber tubing and/or a column of carbon embedded in hydroxyethylcellulose polymer the rabbits hyperventilated, developed a respiratory alkalosis and died. Death in these animals was shown to be associated with polymorphonuclear leucopenia and the sequestration of polymorphonuclear leucocytes in pulmonary blood vessels but not the hemoperfusion device itself. This syndrome was reproduced by infusing rabbits with unheated, but not by preheated, serum which had been perfused through silicone rubber tubing or a column of polymer-embedded carbon. Thus, it seems probable that the interaction of a heat labile factor in plasma, which could be complement, with silicone rubber tubing or polymer embedded carbon was responsible for the pulmonary sequestration of leucocytes and the death of the animals. The role of complement in the pathogenesis of this syndrome is being studied. Hemoperfusion through silastic tubing was not associated with hyperventilation, leukopenia or death of animals. Consequently this tubing has been incorporated into the hemoperfusion devices currently being assessed.

To determine the ability of varous preparations of carbon to remove a variety of substances found in plasma while avoiding bioincompatibility due to direct interaction of carbons with the formed elements of blood and embolization of carbon particles, blood is being perfused over a biocompatible microporous polypropylene membrane (0.1 μ pore size) and the dialysate passed through a column of carbon before recirculating over the membrane. This hemoperfusion system avoids the disadvantages associated with fluid shifts across the microporous membrane. The design of this system involved the evaluation of the most appropriate dialysis membrane in terms of biocompatibility, pore size, porosity and surface area, while minimizing the amount of blood in the extracorporeal circuit.

Three different carbons, suitable for inclusion in this device, were assessed for their ability to extract eleven metabolites from plasma which contained them at concentrations similar to those found in FHF. The carbons were a granular carbon (particle size 0.9-1.4 μ diameter), a sperical carbon (particle size 0.6 mm diameter) and the polymer embedded carbon. Clearance values were determined using plasma supplemented with labeled and unlabeled metabolites in a simple recirculating system which included a carbon column. Each of the three types of carbon utilized were inefficient at extracting dicarboxylic amino acids and short chain fatty acids. The polymer embedded carbon was moderately effective and the other two carbons were very effective in removing aromatic amino acids and neurotransmitters from plasma. Tryptophan, a metabolite avidly cleared by each of the columns in the simple recirculating system was also avidly cleared from plasma perfused through the hemoperfusion system. Furthermore, normal rabbits subjected to hemoperfusion through the system for 4 hour periods have shown no ill effects.

In the light of these encouraging findings, controlled trials of carbon hemoperfusion treatment of the rabbit model of FHF are being conducted. In addition, the reversibility of the model is also being assessed by experiments in which rabbits in FHF are cross-circulated with normal rabbits. Because no objective grading method for depth of encephalopathy in animals has been established, we have begun to explore the use of computer-averaged evoked potentials for this purpose in these experiments. (E. A. Jones, D. F. Schafer, M. L. Zeneroli-Baraldi, J. G. Waggoner, T. Kolobow [NHLBI], R. L. Dedrick [BEI].

V. Studies of Alpha-1-antitrypsin Metabolism and of Protease Inhibitor Phenotypes

We have previously determined pool sizes, fractional catabolic rates, plasma half-lives and synthetic rates of alpha-1-antitrypsin (α_1AT) in normal subjects with the protease inhibitor (Pi) phenotype MM from studies of the turnover of tracer doses of radioiodinated pure undenatured biologically active α_1AT isolated from PiMM subjects (M protein). The turnover data indicated that a constant volume of plasma is cleared of α_1AT per unit time and that α_1AT is catabolized in, or in close relationship to, the intravascular compartment. In common with other plasma glycoproteins, desialylation of α_1AT with neuraminidase was associated with a striking decrease in its plasma half-life due to rapid uptake of the desialylated glycoprotein by the liver.

α_1AT has been isolated from subjects with α_1AT deficiency and the Pi phenotype ZZ (Z protein). Simultaneous metabolic turnover studies of the M and Z proteins are currently being conducted in normal subjects (PiMM) and patients with α_1AT deficiency (PiZZ) using ^{125}I and ^{131}I to label each of the two proteins, respectively. These studies will enable the contribution of decreased hepatic release of α_1AT and increased catabolism of α_1AT to the low serum concentrations of α_1AT in α_1AT deficiency to be assessed and will determine whether the differences in molecular structure between the Z and M proteins influence the rate of catabolism of α_1AT.

Three studies involving Pi phenotyping have been completed. (i) A family study of a proband with the rare Pi phenotype SZ, α_1AT deficiency and cirrhosis with globules of α_1AT material in hepatocytes has been conducted. The proband was shown to have appreciable hepatic copper overload, chronic fibrosing interstitial pneumonitis and, terminally, a hepatocellular carcinoma. (ii) No significant increase in non-MM Pi phenotypes was found in 37 patients with rheumatoid arthritis and 40 patients with systemic lupus erythematosus suggesting that particular Pi types neither predispose to the development nor enhance the severity of these diseases. (iii) No significant increase in non-MM Pi phenotypes was found in 52 patients with Sjogren's syndrome. Mean values for tests of pulmonary function, including estimates of both restrictive lung disease and airway obstruction, were not significantly different for patients with Sjogren's syndrome who had the Pi MM phenotype and those with non-MM Pi phenotypes. (E. A. Jones, J. Vergalla, C. J. Steer, J. Karsh, J. E. Gadek [NHLBI], R. G. Crystal [NHLBI]).

VI. Studies of the Distribution of Receptors for Modified Glycoproteins on Parenchymal and Non-parenchymal Hepatic Cells

Modified glycoproteins bearing terminal non-reducing sugar residues other than sialic acid are rapidly cleared from plasma by the liver. In initial experiments the cell types responsible for the uptake of these glycoproteins by rat liver were determined. For these studies a technique was developed to isolate viable hepatocytes and hepatic sinusoidal cells with intact cell surface membrane receptors. This goal was achieved by employing a combination of gravity sedimentation and Metrizamide density gradient centrifugation. The carbohydrate moiety of the glycoprotein orosomucoid was enzymatically modified *in vitro*. The resulting derivatives, labeled with ^{125}I, were injected intra-

venously. Subsequently, protein-bound radioactivity associated with isolated cell fractions was determined. The data suggested that hepatocytes were responsible for the uptake of galactose-terminated glycoproteins, whereas sinusoidal cells were responsible for the uptake of glycoproteins with terminal N-acetylglucosamine or mannose residues. In complementary in vitro experiments isolated hepatocytes and sinusoidal cells were incubated with each of the ^{125}I-labeled derivatives of orosomucoid. The galactose-terminated derivative was taken up only by hepatocytes, whereas the N-acetylglucosamine and mannose-terminated derivatives were taken up only by sinusoidal cells. Uptake of the mannose-terminated derivative by isolated sinusoidal cells was saturable and could be described by Michaelis-Menten kinetics. The apparent Km was 3.6×10^{-7} M. Carrier dilution studies confirmed the specificity of a galactose recognition system on hepatocytes and a mannose recognition system on sinusoidal cells. To supplement the data obtained using glycoproteins which had been enzymatically modified in vitro, the cellular uptake of an intact glycoprotein which is rapidly taken up by the liver was studied. The glycoprotein chosen was β-hexosaminidase A (βH). The plasma half-life of ^{125}I-βH was markedly prolonged by simultaneously infusing cold βH or mannan but not by infusing a galactose-terminated glycoprotein. In vivo uptake of ^{125}I-βH per sinusoidal cell was approximately 70 times greater than that per hepatocyte. When ^{125}I-βH was incubated with cell fractions in vitro there was appreciable uptake of the labeled ligand by sinusoidal cells but no detectable uptake by hepatocytes. The uptake of ^{125}I-βH and ^{125}I-mannose-terminated orosomucoid by sinusoidal cells exhibited similar kinetics. The apparent Km for βH was 4.3×10^{-8} M. Simultaneous incubation of ^{125}I-βH with galactose-, N-acetylglucosamine-, or mannose-terminated glycoproteins or with mannan resulted in 0, 50, 85 and 98% inhibition of uptake of the labeled ligand by sinusoidal cells. There was markedly diminished uptake of ^{125}I-βH by viable sinusoidal cells which had been exposed to Pronase. These findings suggest that a mannose-specific receptor on sinusoidal cells mediates the hepatic clearance of βH.

In related studies sequential enzymatic modifications of the carbohydrate moiety of glucocerebrosidase in vitro resulted in the generation of derivatives of the enzyme which retained their glucocerebrosidase enzymatic activity. The half-life of glucocerebrosidase activity in the plasma of rats was shorter after infusing derivatives of the enyzme than after infusing the intact enzyme. Isolated sinusoidal cells contained 7 units of glucocerebrosidase activity per 10^6 cells. After infusing 600,000 units of intact glucocerebrosidase and its galactose-, N-acetylglucosamine- and mannose-terminated derivatives isolated sinusoidal cells contained 48, 16, 170 and 260 units of glucocerbrosidase activity per 10^6 cells, respectively. Thus, markedly augmented uptake of infused glucocerebrosidase by sinusoidal cells was achieved by administering the mannose-terminated derivative of the enzyme.

Thus, we have shown that the rapid clearance of galactose-, N-acetylglucosamine- and mannose-terminated glycoproteins from plasma by the liver appears to be receptor-mediated. A galactose-specific receptor is unique to hepatocytes. In contrast, a mannose-specific receptor is localized on sinusoidal cells. The rapid hepatic clearance of at least one lysosomal enzyme, β-hexosaminidase A, appears to be mediated by a sinusoidal cell mannose recognition system. Such a process may be of physiologic importance in preventing the accumulation in the circulation of potentially autodestructive lysosomal enzymes. Finally, the

observation that the cellular distribution of an infused lysosomal glycosidase can be manipulated by specifically modifying its carbohydrate moiety is of potential clinical importance in enzyme replacement therapy. Studies are in progress to determine the number of galactose-specific receptor sites on the hepatocyte, as well as to define the kinetics of uptake of galactose-terminated glycoproteins. (E. A. Jones, C. J. Steer, S. Furbish [NINCDS], J. A. Barranger [NINCDS], R. O. Brady [NINCDS], R. Clarenburg, J. W. Kusiak [NINCDS]).

ANNUAL REPORT SUMMARY
DIGESTIVE DISEASE BRANCH

Phoenix Clinical Research Section

The Section conducts clincial and laboratory research relating to cholelithiasis, diabetes, and its complications primarily in the American Indian populations of the Southwest. Many of the investigations are done in close collaboration with the Southwestern Field Studies Section of the Epidemiology and Field Studies Branch.

Studies of the determinants of bile lithogenicity by the Section have focused in two areas during the past year. Firstly, the effects of estrogens on bile composition have been examined. Differences in the size of bile acid pools between men and women were found with premenopausal females having smaller pools than males of similar age. Secondly, it was found that the use of oral contraceptives resulted in an increase in biliary cholesterol secretion without any consistent pattern of alteration in bile acid pool sizes or bile acid secretion rates. Thus, oral contraceptives were associated with an increase in the lithogenicity of bile and which probably explains the increased rates of cholelithiasis which have been described in women receiving oral contraceptives elsewhere.

Since Pima Indian females have an extraordinarily high frequency of cholelithiasis by the third decade, the composition of gall bladder bile and bile acid pool sizes, determined using deuterated chenodeoxycholic acid, were examined in male and female Pima Indians aged between 9 and 21 years of age. These findings were correlated with measures of growth and puberty. Biliary lithogenicity was found to increase in both Pima males and females during puberty. The major correlates of the increase in lithogenicity, other than age itself, were increasing obesity and urinary estrogen excretion. The results of this investigation suggest that the high rate of gallstone formation and high frequency biliary lithogenicity in the Pima Indians is a characteristic acquired during the teen age years. Lithogenic bile was not found prior to puberty, but following puberty, it was found in greater frequency in females and its frequency correlated with the extent of urinary estrogen excretion. No systematic relationship between bile acid pool size and estrogen excretion was demonstrated. Since the frequency of cholelithiasis increases markedly in the third decade, the data suggests that there is probably an 8 to 10 year lag period between the development of lithogenic bile and the appearance of clinically detectable gallstones.

The characteristics of lipoprotein patterns in Indian and Caucasian diabetics and non-diabetics have been extensively investigated. Diabetes is associated with a decrease in HDL cholesterol levels and an increase in LDL cholesterol levels. Lower total cholesterol and LDL cholesterol levels were found in the Indians, which was associated with decreased LDL synthetic rates in the Indians. While American Indians with diabetes have higher total cholesterol, LDL cholesterol and VLDL triglyceride levels than those without diabetes, the level of each of these lipids was shown to fall significantly following the induction of a remission of diabetes by dietary means with accompanying weight loss. Such remissions were associated with an increase in the ratios

of HDL to LDL cholesterol with an accompanying fall in total cholesterol levels and in triglyceride concentrations. Such changes would appear to be beneficial given that total cholesterol and LDL cholesterol levels are risk factors for the development of macrovascular disease, and that HDL levels or the HDL/LDL cholesterol ratio is negatively correlated with the development of macrovascular atherosclerotic disease. However, the investigation of a small number of American Indians with and without coronary heart disease failed to reveal systematic differences in the directions predicted.

The subfractions of HDL which correlate with ischemic heart disease are currently under study. Since the American Indians and the Pima Indians in particular, have a low rate of coronary artery disease in spite of their obesity and high prevalence of diabetes, the concentration of HDL subfractions will be compared in Pima Indians and in Caucasians.

VLDL metabolism has been investigated to determine the relationship between VLDL and other serum lipoprotein classes and to determine whether the hyperinsulinism previously documented in the Pima Indians influences VLDL metabolism. Incorporation of labeled glycerol into VLDL has been studied to determine synthetic and catabolic rates of VLDL. The results to date indicate that VLDL triglyceride synthesis is significantly reduced in the Pima Indians compared to that of Caucasians of similar degrees of obesity, and that the fractional catabolic rate was significantly higher. Intermediate density lipoprotein fractions have been isolated from these subjects to assist in the development of a multi-compartmental model for IDL metabolism. The further understanding of the reasons for the lower synthetic rates of LDL and VLDL in the Pima Indians, using double labeling techniques, should help elucidate reasons for the relative rarity of coronary artery disease among the Pima Indians, including those with diabetes, and may perhaps shed light on why Caucasian diabetics are so prone to develop atherosclerosis.

Free fatty acid levels have been studied in Pima Indians with severe and mild diabetes and in obese non-diabetic Pimas, and obese non-diabetic Caucasians. It was shown that free fatty acid levels in the Pima Indian diabetics are exquisitely sensitive to insulin at a time when the same subjects are resistant to the glucose lowering effects of insulin. These observations may explain why obesity can be maintained and increased at a time when subjects are becoming glucose intolerant, and indicates how the maturity onset diabetic may be resistant to the glucose lowering effects of insulin, yet may fail to develop any evidence of ketosis due to retention of sensitivity to the antilipolytic effects of extremely small amounts of circulating or exogenous insulin.

Investigation of the mechanisms underlying remission of diabetes which accompanies carbohydrate restriction and weight loss have been continued. Recent onset diabetics show a selected loss of glucose induced insulin secretion at a time when they retain considerable responsiveness to amino acid stimulation. These findings indicate that one component of the diabetic state in the maturity onset diabetic is a loss of the glucose sensing capacity of the pancreas at a time when the insulin secreting capacity and responsiveness to other stimuli is substantially intact.

Weight reduction and carbohydrate restriction have been shown to be associated with a substantial increase in insulin responsiveness to an oral glucose load at a time when fasting glucose levels are essentially normal, but post load levels remain elevated. Since continuation of weight loss then results in a continuing improvement in glucose tolerance which is accompanied by a fall in the post challenge hyperinsulinemia, and since obese subjects without diabetes, but who are glucose intolerant, have a similar post load hyperinsulinemia which also diminishes markedly with weight loss, it is postulated that an important step in the development of maturity onset diabetes is the development of increasing resistance to the glucose lowering effects of insulin.

Studies of insulin receptors in the Pima Indians have shown that while specific binding is reduced in both the obese and obese diabetic, weight reduction is accompanied by an increase in specific binding in both groups to a similar degree. It is postulated, therefore, that the obese diabetic shows increased insulin resistance which is not primarily the result of an insulin receptor deficiency. This suggests that the basis of insulin resistance in obesity and diabetes is perhaps an intracellular abnormality, although the reduced specific binding of insulin to cellular receptors probably contributes secondarily to the likelihood of decompensation of glucose metabolism.

Measurement of in-vivo insulin resistance using insulin clamp methods has shown that insulin resistance increases with both obesity and fasting hyperinsulinemia. Nevertheless, comparison of the degree of insulin resistance in subjects with impaired glucose tolerance and in obesity matched subjects with normal oral glucose tolerance, has shown that the degree of insulin resistance is greater in the subjects with glucose intolerance. These studies, in conjunction with the changes which have been observed during the induction of remissions in diabetes, strongly suggest that the development of insulin resistance is a key step in the development of diabetes and that those who manifest glucose intolerance have a greater degree of insulin resistance than those whose glucose tolerance remains normal.

Hyperinsulinemia which has been previously shown to be a characteristic of non-diabetic Pima Indians, when compared to Caucasians of similar degrees of obesity, could be the result of differences in the degrees of insulin resistance between the two groups, or differences in the fractional extraction of insulin. To determine whether differences in the fractional extraction of insulin were present between Pima Indians and Caucasians urinary C-peptide levels were measured and showed that there were no differences in the fractional extraction of insulin in either Pimas or Caucasians of equivalent degrees of obesity. Furthermore, obesity itself was not associated with differences in fractional insulin extraction rates.

Investigations have been initiated to determine the cellular basis of insulin resistance in the Pima Indians. For many of these studies the cultured fibroblast has been selected for investigation. Suitable cell lines have been established from normal and diabetic Pima Indians and the parameters of cell growth between the normal and diabetic Pima cells have

been found not to differ. However, a systematic difference between the growth characteristics of Pima cells and Caucasian cells has been identified, but it is postulated that this difference has an ethnic basis, rather than being related primarily to the determinants of diabetes. Abnormalities in the ability of these cells to down regulate their insulin receptors in response to incubation with insulin have been documented. Such a failure is consistent with similar findings on circulating monocytes in-vivo. The insulin receptors on these cells have been characterized in relation to their number and affinity, and methods for solubilizing and purifying insulin receptors from diploid fibroblasts are currently being established.

Physiologic concentrations of insulin have been shown to stimulate glucose incorporation in the cultured fibroblasts and kinetic studies have shown that glucose starvation stimulates the V-max but not the Km of the hexose transport system. Studies of the fate of incorporated glucose indicate that about 30% is converted to glycogen, 30% remains in the acid soluble portion of the cell, predominantely as nucleotides, and 40% is converted to lactate. While the transport of hexose in these cells has been shown to involve a large diffusion component, insulin causes a small, but significant increase in V-max without influencing the Km.

Pima and Caucasian cells have been compared with the respect to the stimulation glucose uptake by physiologic levels of insulin. No differences between the non-diabetic or diabetic Pima cells or between the Pima and Caucasian cells have been demonstrated. The system has also been used to demonstrate that insulin can stimulate glycogen synthase activity in cultured fibroblasts. Future studies will incorporate studies of insulin binding, receptor regulation, hexose transport and other intracellular responses to insulin. It is hoped that these will lead to an understanding of the mechanism of insulin resistance and its cellular basis.

Preliminary data has been obtained on $Na^+ + K^+$ ATPase activity in red cells from normal and obese Caucasians and obese Pima Indians. Obese Pimas appear to have a mean level of activity which is significantly lower than Caucasian. These studies will be pursued and $Na^+ + K^+$ ATPase activity will be measured in diploid fibroblasts, since confirmation of these differences, may provide a possible explanation for the development of obesity in such high frequency among the Pima Indians.

Glycosylated hemoglobin concentrations have been measured in a large number of Pima Indians with a wide spectrum of age and glucose tolerance in conjunction with the ongoing epidemiological studies of the Southwestern Field Studies Section. The frequency distribution of glycosylated hemoglobin levels in Pima Indians age 25 and over was shown to be bimodal. The relationship between glycosylated hemoglobin levels and the bimodal frequency distributions of the fasting and two hour post load plasma glucose levels in the same subjects showed a high degree of concordance between the elevation of glycosylated hemoglobin levels and both fasting and two-hour glucose levels. The characteristics of changing glycosylated hemoglobin levels with increasing age has been documented. The relationship between the specific complications of diabetes and glycosylated hemoglobin level have been examined and, as expected, there was a high correlation between

elevated glycosylated hemoglobin level and the presence of complications, but no evidence has been found that glycosylated hemoglobin levels discriminate better than the fasting or two-hour glucose measurements for this purpose. Whether or not glycosylated hemoglobin levels predict the future development of complications more effectively than the glucose levels, however, remains to be determined. Nevertheless, glycosylated hemoglobin provides an index of glycemia which may be useful in epdemiological studies where the performance of a glucose tolerance test or the determination of glucose levels in the fasting state is impractical.

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PERIOD COVERED

October 1, 1978 to Sept. 30, 1979.

TITLE OF PROJECT (80 characters or less)

Studies of Membrane Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. D. Gardner	Chief, Section on Gastroenterology	DDB, NIAMDD
OTHERS:	M. Jackson	Guest Worker	DDB, NIAMDD
	S. Batzri	Visiting Associate	DDB, NIAMDD
	R. T. Jensen	Senior Investigator	DDB, NIAMDD
	B. Tai	Guest Worker	DDB, NIAMDD
	T. Q. Garvey	Guest Worker	DDB, NIAMDD
	P. Hyman	Clinical Associate	DDB, NIAMDD
	C. Costenbader	Guest Worker	DDB, NIAMDD
	H. Binder	Guest Worker	DDB, NIAMDD
	S. Collins	Visiting Scientist	DDB, NIAMDD
	L. Korman	Clinical Associate	DDB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	1.6	1.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The broad categories which are included in the project are:
1) Characterizing functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) identifying the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) developing techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane; 4) characterizing the mechanism by which the membrane transport of various substrates is altered in certain diseases; and 5) relating these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

PHS-6040
(Rev. 10-76)

In acinar cells from guinea pig pancreas enzyme secretion can be increased by at least 15 different naturally occurring secretagogues. During this past year we have characterized the kinetics of the "basal secretory state" and of the "stimulated secretory state". The kinetics of the stimulated state differs substantially depending on whether the secretagogue acts by activating adenylate cyclase or by causing release of membrane-bound calcium. We have also found that in addition to acting as a mediator of the action of certain secretagogues, cellular calcium also functions to modulate one or more of the final steps in the secretory process.

The initial step in the mechanism of action of pancreatic secretagogues is reversible binding to a receptor located on the outer surface of the plasma membrane. We have used ^{125}I-[Tyr^{4}]bombesin to identify receptors that interact with bombesin, litorin, ranatensin and alytesin and have shown that maximal stimulation of enzyme secretion occurs when 45% of the receptors are occupied. We have also found that short chain alcohols can modify the function of secretagogue receptors in acinar cells, probably by increasing membrane fluidity.

Publications:

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Jensen, R. T. and Gardner, J. D. Receptors for secretagogues on pancreatic acinar cells: Different mechanisms of spareness. In Bonfils, S., Fromageot, P. and Rosselin, G. (Ed.): Hormone Receptors in Digestion and Nutrition. North Holland, Elsevier/North-Holland Biomedical Press, (In Press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53002-07 DDB

PERIOD COVERED

October 1, 1978 to Sept. 30, 1979

TITLE OF PROJECT (80 characters or less)

Gastrointestinal Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. D. Gardner	Chief, Section on Gastroenterology	DDB, NIAMDD
OTHER:	M. Jackson	Guest Worker	DDB, NIAMDD
	S. Batzri	Visiting Associate	DDB, NIAMDD
	D. M. McCarthy	Senior Investigator	DDB, NIAMDD
	R. T. Jensen	Senior Investigator	DDB, NIAMDD
	B. Tai	Guest Worker	DDB, NIAMDD
	P. Hyman	Clinical Associate	DDB, NIAMDD
	C. Costenbader	Guest Worker	DDB, NIAMDD
	L. Korman	Clinical Associate	DDB, NIAMDD
	N. Barlas	Visiting Scientist	DDB, NIAMDD
	S. Collins	Visiting Scientist	DDB, NIAMDD
	S. Abdelmoumene	Guest Worker	DDB, NIAMDD
	S. Pandol	Clinical Associate	DDB, NIAMDD

COOPERATING UNITS (if any)

Dept. of Chemistry, Case-Western Rese rve Univ. Cleveland, Ohio
Div. of Cellular Biology, Kennedy Institute for Rheumatology, London, England

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro systems are being used to study the mechanism of action of gastrin, secretin, cholecystokinin and vasoactive intestinal peptide with their specific membrane receptors.

Clinical investigations are directed toward developing alternative forms of therapy for and elucidating the pathogenesis of disorders characterized by ectopic production of gastrointestinal hormones (e.g., Zollinger-Ellison syndrome and pancreatic cholera).

PHS-6040
(Rev. 10-76)

Dispersed acinar cells from guinea pig pancreas possess two functionally distinct classes of receptors each of which cause activation of adenylate cyclase and increase cyclic AMP when occupied by VIP or secretin. One class of receptors has a high affinity for VIP and a low affinity for secretin; the other has a low affinity for VIP and a high affinity for secretin. These two classes of receptors can also be distinguished by their abilities to distinguish among different C-terminal fragments and analogues of secretin and VIP.

In addition to cholecystokinin and cholinergic agents the following peptides have been found to increase enzyme secretion from pancreatic acinar cells by causing release of membrane-bound calcium: caerulein, physalaemin, substance P, eledoisin, kassinin, bombesin, litorin, alytesin, ranatensin.

In dispersed acinar cells from guinea pig pancreas, VIP and secretin interact with a common membrane receptor, activate adenylate cyclase and increase cellular cyclic AMP. In these same cells cholecystokinin and cholinergic agents cause release of membrane-bound calcium, increased cellular cyclic GMP and stimulation of amylase secretion.

In patients with Zollinger-Ellison syndrome the histamine H_2-blocking agent, Cimetidine, will abolish gastric acid secretion and, as a result, is a therapeutic alternative to total gastrectomy.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53004-07 DDB

PERIOD COVERED
October 1, 1978 to Sept. 30, 1979

TITLE OF PROJECT (80 characters or less)

Cyclic Nucleotide Mediated Functions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. D. Gardner	Chief, Section on Gastroenterology	DDB NIAMDD
OTHER:	M. Jackson	Guest Worker	DDB NIAMDD
	S. Batzri	Visiting Associate	DDB NIAMDD
	R. T. Jensen	Senior Investigator	DDB NIAMDD
	B. Tai	Guest Worker	DDB NIAMDD
	P. Hyman	Clinical Associate	DDB NIAMDD
	C. Costenbader	Guest Worker	DDB NIAMDD
	L. Korman	Clinical Associate	DDB NIAMDD
	S. Collins	Visiting Scientist	DDB NIAMDD
	N. Barlas	Visiting Scientist	DDB NIAMDD
	S. Abdelmoumene	Guest Worker	DDB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Gastroenterology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vitro systems are being used to characterize the mechanism by which cyclic nucleotides alter cell function and to explore the mechanism of action of agents whose effect on cell function is mediated by cellular accumulation of cyclic nucleotides.

PHS-6040
Rev. 10-76)

Peptides isolated from amphibian skin possess the abilities to activate guanylate cyclase and increase cellular cyclic GMP in pancreatic acinar cells as well as cells from other gastrointestinal tissues. This ability to increase cyclic GMP is modulated by the cellular calcium content in that reducing cellular calcium causes a corresponding reduction in cellular cyclic GMP.

In dispersed parietal cells prepared from guinea pig stomach the action of histamine is produced by its interaction with histamine H_2-receptors and mediated by cellular cyclic AMP. There is a good correlation between the abilities of various agents to antagonize the binding of histamine and their abilities to inhibit gastric acid secretion in vivo and in vitro.

All of the agents which are known to cause secretory diarrhea in man are thought to do so by activating adenylate cyclase and increasing cellular cyclic AMP. Inhibitors of cyclic nucleotide phosphodiesterase cause a worsening of secretory diarrhea, whereas agents such as lithium which reduce the activity of adenylate cyclase reduce secretory diarrhea.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,500-06 DDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (30 characters or less)
Studies of the Transport of Organic Anions by the Liver

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Sec. Dis. of Liver	DDB:NIAMDD
Other:	J. G. Waggoner	Chemist	DDB:NIAMDD
	W. B. Jakoby	Chief, Sec. Enzyme and Cell Biol.	LBM:NIAMDD

COOPERATING UNITS (if any)

LBM:NIAMDD

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	1/2	1/4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The hepatic uptake and net hepatic clearance of conjugated bilirubin in rats is substantially faster than that of unconjugated bilirubin. An appreciable affinity of conjugated bilirubin for intrahepatic binding proteins, such as ligandin, has been demonstrated. The existence of a significant intrahepatic storage pool for bilirubin monoglucuronide, but not bilirubin diglucuronide, has also been established. These observations may imply that the formation and excretion of bilirubin diglucuronide occurs directly from the canalicular pool of the hepatocyte. Saturable binding of sulfobromophthalein to hepatic canalicular and plasma membranes has been demonstrated. Furthermore, proteins with affinities for bilirubin and BSP have been isolated from hepatic cell surface membrane fractions. In studies of bile salt transport it has been shown that microfilaments play a role in hepatic active transport systems and that the excretion of currently unidentified anions contribute to the bile salt independent fraction of bile.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To study the transport of organic anions from blood across the hepatocyte into bile, in order to characterize the biophysical nature of each of the transport steps involved in the overall hepatic excretory system.

2) To develop a capability for quantitating the function of each of these steps in patients with hepatic dysfunction by analysis of kinetic studies of various test substances such as sulfobromophthalein, indocyanine green, radiolabeled bilirubin and radiolabeled bile salts.

Methods Employed:

1) Plasma disappearance curves of labeled or unlabeled organic anions are analyzed using appropriate computer programs.

2) Pure preparations of biosynthetically labeled bilirubin conjugates are isolated from rat bile using albumin-agarose affinity columns.

3) Homogenates and cytosol of liver are subjected to thin layer chromatographic analysis of ^{3}H-labeled bile pigments after administrating unconjugated ^{3}H-bilirubin intravenously to rats. The dissociation constants of bilirubin and its conjugates for glutathione-S-transferase are measured fluorometrically.

4) Rat liver plasma and canalicular membrane fractions are prepared by differential and isopycnic centrifugation.

5) Proteins with affinities for sulfobromophthalein and bilirubin are isolated from rat liver membrane fractions by affinity chromatography.

6) A rapid filtration technique is used to separate cells from medium in studies of the initial uptake velocity of taurocholate by hepatocytes.

7) Polyacrylamide gel electrophoresis is used in the assessment of the effect of bile salts on protein excretion into bile.

Major Findings:

1) Fasting and postpranial serum bile acid concentrations and plasma disappearance rates of injected cholyl-glycine are entirely normal in all three subtypes of Gilbert's syndrome. These findings confirm previously fragmentary information which suggests that the transhepatic transport of bile acids occurs over separate pathways from those involved in the excretion of other cholephilic anions. They are also of clinical significance in that even minimal structural hepatic disease has been found almost invariably to be associated with an abnormality in either the postprandial bile acid concentration or plasma bile acid disappearance rates. Hence, in patients

with subtypes of Gilbert's syndrome associated with abnormalities of BSP metabolism the presence of normal bile acid studies strongly mitigates against the existence of structural liver disease and can be used as a means of obviating the necessity for invasive procedures such as liver biopsy in these patients.

2) An analysis of plasma disappearance curves of injected cholyl-glycine in normal subjects as assessed by a radioimmunoassay, which was reported by others, resulted in values for the initial volume of distribution that exceeded that of total body water and values for clearance from the plasma which exceeded hepatic blood flow. These findings prompted a further study of the disappearance of injected cholyl-glycine from plasma in normal subjects in which measurements of cholyl-glycine were made using the 3-hydroxysteroid dehydrogenase method. This study indicated that the rate of disappearance of unlabeled cholyl-glycine from plasma was the same as that of ^{14}C-labeled cholyl-glycine. This rate was similar to the corresponding rate for the more extensively studied cholephilic anion indocyanine green and calculated values for the initial volumes of distribution appoximated to the plasma volume.

3) To obtain new insights into the relationship between plasma bilirubin turnover and hepatic bilirubin clearance, studies of bilirubin kinetics have been conducted in patients with hereditary spherocytosis before and after splenectomy. In patients whose pre-operative bilirubin clearance values were normal, the fall in bilirubin production occurring after splenectomy had no effect on hepatic bilirubin clearance, whereas in patients whose pre-operative bilirubin clearance values were low and in the range found in Gilbert's syndrome, there was a marked increase in hepatic bilirubin clearance following surgery. These findings suggest that in patients with Gilbert's syndrome the hepatic bilirubin transport system is operating at a level close to saturation and that bilirubin loading studies may prove to be a useful provocative test for the diagnosis of carriers of the Gilbert's syndrome gene who do not express the trait at basal levels of bilirubin production.

4) The previous isolation of a pure preparation of biosynthetically labeled bilirubin conjugates in this laboratory has enabled kinetic studies of the hepatic metabolism of conjugated bilirubin to be carried out in rats. It has been shown that values for both hepatic uptake and net hepatic clearance of conjugated bilirubin are substantially faster than corresponding values for unconjugated bilirubin, presumably due, at least in part, to the ability of the former pigment to bypass the conjugation step. In addition, the hepatic uptake of conjugated bilirubin, like that of the unconjugated pigment, was inhibited by other organic anions, such as BSP and indocyanine green, but not by glycocholate, suggesting that the hepatic uptake of conjugated bilirubin is not mediated by simple diffusion but by a more complex mechanism shared with unconjugated bilirubin.

5) Bilirubin monoglucuronide accumulates preferentially within the hepatocyte whereas the diglucuronide appears to be preferentially secreted

into bile. In addition, unconjugated and conjugated bilirubins were found to have a high affinity for glutathione-S-transferases such as ligandin. Based on these findings it is suggested that microsomal monoconjugation of bilirubin may occur while it is bound to a glutathione-S-transferase and that the biliary excretion of bilirubin may involve interaction of a bilirubin monoglucuronide glutathione-S-transferase complex with the bile canalicular membrane with resultant excretion of bilirubin diglucuronide directly from the canalicular pool of the hepatocyte.

6) To assess the potential role of membrane carriers in the hepatic transport of organic anions the kinetics of binding of labeled sulfobromophthalein (BSP) to canalicular and plasma membrane fractions isolated from rat hepatocytes were studied. Saturable binding of BSP to both membranes was shown and this binding was competitively inhibited by bilirubin and indocyanine green but not by taurocholate. This investigation has provided further support for the hypothesis that organic anions other than bile salts share a common pathway for hepatic uptake and biliary excretion and that membrane carrier proteins may be involved in these processes.

7) An attempt has been made to isolate potential carrier proteins from sinusoidal and canalicular enriched liver membrane fractions using affinity chromatography. From BSP affinity columns three binding proteins with apparent molecular weights of 4.0, 4.6 and 6.0 x 10^4 were eluted. From bilirubin affinity columns a single binding protein with an apparent molecular weight of 6.0 x 10^4 was eluted. Thus, one or more membrane carrier proteins may be involved in the transport of bilirubin and organic anion dyes.

8) To investigate whether elevated fasting and post-prandial serum bile salt concentrations in patients with chronic liver disease might be due, at least in part, to abnormal bile salt transport, uptake of taurocholate by hepatocytes isolated from normal rats and rats with fibrosis induced by carbon tetrachloride was determined. Taurocholate uptake by hepatocytes from rats with hepatic fibrosis was noncompetitively inhibited, raising the possibility of decreased numbers of receptors for bile salts on these cells.

9) In a study of the differential effects of taurolithocholate and taurocholate on protein excretion into bile in rats, it has been shown that taurocholate induces some loss of surface proteins of the canalicular membrane without affecting the integrity of this membrane. In contrast, taurolithocholate induced substantially more protein excretion and this was associated with loss of integral canalicular membrane and Golgi components into bile. These findings may be related to the observed loss of canalicular microvilli and cholestasis caused by taurolithocholate.

10) The role of microfilaments and microtubules in bile salt transport has been studied in rats by assessing the effects of cytochalasin B, a microfilament inhibitor, and colchicine, a microtubule inhibitor, on taurocholate uptake by isolated rat hepatocytes. Cytochalasin B competitively inhibited taurocholate uptake, implying either binding of cytochalasin B to a receptor for taurocholate or direct participation of microfilaments in taurocholate uptake. Colchicine noncompetitively inhibited taurocholate uptake

suggesting either an indirect or no role of microtubules in taurocholate transport. Thus, microfilaments may be importance in active transport systems of the hepatocyte and their functional impairment may lead to cholestasis by inhibiting bile salt transport.

11) In a further study of bile salt transport, an explanation of the anion gap in rat bile was sought. Bile salt-depleted bile was found to increase hepatocellular bile flow in a dose-dependent fashion without altering the permeability of the biliary tree. The unidentified choleretic agent was heat stable and was not a bile salt. This finding suggests that part of the bile salt independent fraction of bile may be dependent on the excretion of currently undefined anions.

Significance to Biomedical Research:

Studies of organic anion kinetics in patients with Gilbert's syndrome, Crigler-Najjar syndrome and normal volunteers have led to a marked improvement in our understanding of the excretory function of the liver. In particular, these studies have demonstrated that it is possible to quantitate hepatic function in terms of clearance values in a manner analagous to the use of creatinine clearance measurements for quantitating renal function. The ability to accurately measure both bilirubin turnover and bilirubin clearance, the two parameters which determine the plasma bilirubin concentration, has made possible a precise functional classification of all cases of unconjugated hyperbilirubinemia. Studies of the sort described above are also leading both to the development of new clinical methods for assessing the function of individual steps in organic anion transport processes and to an improved understanding of the nature of the defects in a variety of disease states.

Proposed Course:

This project is currently inactive. Future studies might include the investigation of new approaches to the quantitation of bilirubin kinetics which involve the intravenous injection of various loading doses of bilirubin, in addition to tracer doses. This approach could be employed in an attempt to determine whether those patients with Gilbert's syndrome, who have demonstrated abnormalities in the hepatic uptake of indocyanine green and sulfobromophthalein, also have a corresponding defect in the hepatic uptake of bilirubin. The methods currently used for the studies of unconjugated bilirubin kinetics in man might be extended to permit studies of the metabolism of conjugated bilirubin in common disease states characterized by conjugated hyperbilirubinemia such as large duct biliary obstruction, cirrhosis and viral hepatitis.

Publications:

1. Scharschmidt, B.F., Martin, J.F., Plotz, P.H., Shapiro, L.J., and Berk, P.D.: Medical applications of affinity chromatography. In Pye, E.K. and Weetall, H.H. (Eds.): Enzyme Engineering. New York, Plenum Publishing, 1978, pp. 427-429.

2. Shupeck, M., Wolkoff, A.W., Scharschmidt, B.F., Waggoner, J.G., and Berk, P.D.: Studies of the kinetics of purified bilirubin-^{3}H in the rat. Amer. J. Gastroenterology 70: 259-264, 1978.

3. Klos, C., Paumgartner, G., and Reichen, J.: Cation-anion gap and choleretic properities of rat bile. Amer. J. Physiol. 236: E434-E440, 1979.

4. Carson, E.R., and Jones, E.A.: Use of kinetic analysis and mathematical modeling in the study of metabolic pathways in vivo: Applications to hepatic organic anion metabolism. Parts I and II. N. Engl. J. Med. 300: 1016-1027 and 1078-1086, 1979.

5. Jones, E.A., Vierling, J.M., Steer, C.J., and Reichen, J.: Cell surface receptors in the liver. In Popper, H., and Schaffner, F. (Eds.): Progress in Liver Diseases, Volume VI. New York, Grune and Stratton, 1979, pp. 43-80.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 53,501-06 DDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Use of Artificial Support Devices for the Treatment of Fulminant Hepatic Failure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Sec. Dis. of Liver	DDB:NIAMDD
Other:	D. F. Schafer	Clinical Associate	DDB:NIAMDD
	L. M. Zeneroli-Baraldi	Visiting Scientist	DDB:NIAMDD
	J. G. Waggoner	Chemist	DDB:NIAMDD
	T. Kolobow	Chief, Pul. Car. Assist Dev. Sec.	IR:TD:NHLBI
	R. L. Dedrick	Chief, Chem. Engineering Sec.	R:BEI

COOPERATING UNITS (if any)

Pulmonary and Cardiac Assist Devices Section, IR, TD, NHLBI
Chemical Engineering Section, BEI, R

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An animal model of acute hepatic failure which closely resembles human fulminant hepatic failure has been developed in rabbits with a view to testing the effectiveness of hemoperfusion through columns of various adsorbents as a means of providing temporary hepatic support. The model induced has the advantages of being (i) non-hazardous to personnel, (ii) reproducible, (iii) potentially reversible and (iv) closely resembling human fulminant hepatic failure in its clinical, electroencephalographic, serum biochemical and hepatic histological features. The ability of columns containing various adsorbents to extract potentially encephalopathic substances from plasma is being assessed. To overcome problems of biocompatibility a hemoperfusion system has been devised in which blood is perfused over a microporous polypropylene membrane and the dialysate passed through a column of adsorbent before recirculating over the membrane.

Project Description:

Objectives:

To develop artificial extracorporeal methods for removing from blood those metabolic products which are potentially encephalopathic and which are ordinarily extracted by the liver. The ultimate objective of this project is the development of an artificial support system for use in the management of acute potentially-reversible hepatic failure.

Methods Employed:

1) To generate an animal model of fulminant hepatic failure, the selective hepatotoxin galactosamine-HCl is administered intravenously according to a precise dose schedule to an inbred strain of rabbits.

2) The clearance (ml/min) of potentially encephalopathic metabolites by columns containing a variety of adsorbents is being measured using plasma supplemented with labeled and unlabeled metabolites in a simple recirculation system.

3) Rabbits in hepatic failure are treated by passing blood over a biocompatible microporous polypropylene membrane and passing the dialysate over a column of an adsorbent before recirculating it over the membrane.

Major Findings:

1) A galactosamine-induced animal model of fulminant hepatic failure has been generated in the rabbit which has the advantages of being (i) non-hazardous to personnel, (ii) reproducible and (iii) potentially reversible. In addition, it closely resembles human fulminant hepatic failure in its clinical, electroencephalographic, serum biochemical and hepatic histologic features.

2) Arousal of patients from coma due to fulminant hepatic failure may be attributable, at least in part, to depletion of protein-bound substances toxic to the brain from a slowly exchanging extravascular pool such as the brain or CSF. The hypothetical effects of different hemoperfusion schedules on the kinetics of depletion of a protein-bound substance, unconjugated bilirubin, have been simulated using a computer. Data on the kinetics of unconjugated bilirubin in man and experimentally determined values for the efficiency of hemoperfusion through albumin-agarose columns in removing unconjugated bilirubin from plasma were used in the simulations. The results suggest that simple alterations in the schedule of hemoperfusions might accelerate depletion of toxins from an extravascular pool and hence promote earlier arousal and possibly more frequent survival in patients with fulminant hepatic failure.

3) Columns containing various activated carbons were ineffecient at clearing dicarboxylic amino acids and short chain fatty acids from plasma. In contrast, columns containing granular and spherical carbons were very

effective at clearing from plasma aromatic amino acids and certain neurotransmitters, substances of potential importance in the pathogenesis of hepatic coma. Columns containing Dowex 20, Dowex 50, BioBead SM-2 and LH-20 are ineffective at removing a representative series of potentially encephalopathic metabolites.

4) Normal rabbits, subjected to hemoperfusion schedules in which blood is perfused through silicone rubber tubing or over columns of carbon embedded in hydroxyethylcellulose developed hyperventilation and respiratory alkalosis and subsequently died. This syndrome is associated with polymorphonuclear leukopenia and the sequestration of polymorphonuclear leukocytes in pulmonary blood vessels. A heat labile factor in plasma, which may be complement, is necessary for the development of this syndrome. Hemoperfusion through silastic tubing is not associated with this syndrome.

5) A hemoperfusion system has been devised which avoids problems of bioincompatibility due to interaction between formed elements of the blood and adsorbents and embolization of particles of adsorbent. Blood is perfused over a microporous polypropylene membrane. The dialysate, which is in a closed circuit, is recirculated over the membrane after passage through a column of adsorbent. This arrangement avoids disadvantages associated with fluid shifts across the membrane. Normal rabbits subjected to 4-hour periods of hemoperfusion using this system have shown no ill effects.

Significance to Biomedical Research:

The mortality rate from fulminant hepatic failure of various etiologies in man is about 80-90%. Various types of temporary hepatic support have been used in the treatment of this syndrome in the hope of buying time to allow hepatic regeneration to take place. Such attempts at taking over the functions of a failing liver have, to date, had little if any effect on the mortality rate. Advances in this field are impeded by continuing uncertainties regarding the pathogenesis of coma in fulminant hepatic failure. It is not clear whether coma is due to toxic substances which impair cerebral function or to the lack of a hepatic synthesized substance which is necessary for normal cerebral function. Furthermore, there are no reliable clinical or laboratory findings which enable the clinician to identify those patients in coma from fulminant hepatic failure who will die irrespective of any treatment modality, those who will recover spontaneously and those whose life might be saved by providing temporary hepatic support. It seems likely that further insights into the pathogenesis of fulminant hepatic failure and meaningful evaluations of the most appropriate treatment modalities for the syndrome may be provided by carefully controlled studies using an animal model of fulminant hepatic failure. Such studies should include correlations between the ability of specific adsorbents to remove particular substances or classes of substances and the effects of hemoperfusion over these adsorbents on an animal model of fulminant hepatic failure.

Proposed Course:

The reversibility of the galactosamine-induced animal model of fulminant hepatic failure is being assessed by experiments in which rabbits in

fulminant hepatic failure are cross-circulated with normal rabbits. Controlled trials of carbon hemoperfusion treatment of the animal model are being conducted. Because no objective method of grading the depth of encephalopathy in animals has been established, the use of computer averaged evoked potentials for this purpose is being assessed.

Publications:

1. Berk, P.D., and Popper, H.: Fulminant hepatic failure. Amer. J. Gastroenterology 69: 349-400, 1978.

2. Zeneroli, M.L., Waggoner, J.G., Schafer, D.F., Kolobow, T., and Jones, E.A.: Development of an extracorporeal hepatic support device: Clearance of amino acids, short chain fatty acids and neurotransmitters by different carbons. In Capocaccia, L., Giunchi, G., and Fischer, J.E.: Amino Acids and Hepatic Encephalopathy, 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,503-05 DDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Immunologic Studies in Chronic Active Hepatitis and Primary Biliary Cirrhosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Sec. Dis. of Liver	DDB:NIAMDD
Other:	S. P. James	Clinical Associate	DDB:NIAMDD
	C. J. Steer	Clinical Associate	DDB:NIAMDD
	W. Strober	Senior Investigator	MET:NCI
	C. O. Elson	Expert	MET:NCI
	M. M. Frank	Clin. Dir., NIAID	LCI:NIAID

COOPERATING UNITS (if any)
Metabolism Branch, NCI
Laboratory of Clinical Investigation, NCI

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 3/4	1 1/2	1/4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Abnormal immune mechanisms are being studied in patients with HB_sAg negative chronic active hepatitis and primary biliary cirrhosis (PBC) selected according to strict diagnostic criteria. A detailed immunologic evaluation is undertaken. The in vitro cytotoxic capacity of lymphocytes and lymphocyte subpopulations (T, K and B cells) against several liver-specific and non-hepatic target cells in tissue culture is evaluated. In addition, the capacity of the reticuloendothelial system to clear particles from the circulation by non-specific phagocytosis and immunospecific mechanisms is being assessed. Finally, T cell mediated help and suppression of pokeweed-mitogen induced immunoglobulin synthesis is studied in vitro using radioimmunoassays to measure IgG and IgM. Results of these studies include the demonstration in PBC of (i) reduced spontaneous cytotoxic function of a subpopulation of lymphocytes which lack surface immunoglobulin but bear Fc receptors; (ii) impaired C3b-receptor mediated clearance function of Kupffer cells and (iii) diminished capacity of T cells to inhibit immunoglobulin synthesis in vitro.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To evaluate in detail the immunologic status of patients with chronic active hepatitis and primary biliary cirrhosis (PBC) with special emphasis on immunologic phenomena currently believed to be of pathogenic significance in these diseases. The ultimate objective is to identify the major pathogenic mechanisms in both diseases.

Methods Employed:

Criteria for patient selection include consistent history, physical examination, results of serum biochemical tests, serum non-organ specific antibody titers and hepatic histology. Patients with a history of ingestion of hepatotoxic drugs are excluded. To date the study has been be limited to HBsAg negative subjects. A few patients with alcoholic liver disease have been studied to generate control data in a chronic liver disease in which the etiology is not primarily immunologic. Similarly, a few patients with large duct biliary obstruction and sclerosing cholangitis have been studied to generate control data in patients who have cholestasis not due to primary biliary cirrhosis.

1) The immunologic status of patients is assessed by multiple intradermal antigen tests, primary immunization with keyhole limpet hemocyanin and pneumococcal polysacchride with subsequent keyhole limpet hemocyanin skin testing, quantitation of T and B cell populations using rosetting and immunofluorescence techniques, quantitation of serum concentrations of immunoglobulins and functional (hemolytic) assay of complement components. Patients are also HLA typed.

2) The cytotoxic capacity of lymphocytes and lymphocyte subpopulations harvested from peripheral blood is assessed in an *in vitro* cytotoxicity assay, using target cells which include Chang human hepatoma cells and mouse EL-4 sarcoma cells. The assay depends on quantitating ^{51}Cr released from labeled target cells by lymphocytes from normal subjects and patients run in parallel. The technique is used to assay spontaneous cell-mediated cytotoxicity, mitogen induced cellular cytotoxicity (using phytohemagglutinin, concanavalin A and pokeweed mitogen) and antibody dependent cellular cytotoxicity (using an appropriate anti-target cell antibody).

3) The capacity of the reticuloendothelial system to remove from the circulation particles by non-specific phagocytosis and immunospecific mechanisms is assessed. Nonspecific phagocytosis is determined from the rate of disappearance of a test dose of ^{125}I-microaggregated albumin from plasma and hepatic blood flow from the rate of disappearance of a tracer dose of this substance from plasma. Immunospecific clearance is investigated by following the fate of intravenously administered autologous red cells labeled with ^{51}Cr and coated with a pure IgM or IgG antibody directed against ABO or Rh antigens, respectively. Clearance of IgM coated cells occurs largely in the liver and depends on the interaction between C3b deposited *in vivo* on the red cell membrane and specific C3b receptors on Kupffer cells, while clearance of IgG

coated cells depends on Fc-receptor-bearing fixed macrophages in the spleen. After clearance IgG-coated cells undergo phagocytosis, whereas IgM-coated cells either undergo phagocytosis or release back into the circulation as C3d-coated cells as a result of the action of circulating C3b inactivator.

4) The regulation of immunoglobulin synthesis by B cells is studied in vitro by quantitating T cell mediated help and suppression of pokeweed mitogen-induced immunoglobulin synthesis in vitro. This is achieved by culturing B cells with unirradiated or irradiated T cells at different T:B cell ratios and measuring IgG and IgM synthesis by the cultured B cells using sensitive radioimmunoassays. Immunoglobulin synthesis by autologous and allogeneic mixtures of T and B cells from patients and control subjects are studied.

Major Findings:

Results to date include the following:

1) There is a higher incidence of skin test anergy in patients with primary biliary cirrhosis than in patients with chronic active hepatitis and controls. This anergy includes the response to de novo immunization with keyhole limpet hemocyanin.

2) The cytotoxic response in patients with primary biliary cirrhosis and HBsAg negative chronic hepatitis is not specific for the Chang cell and does not indicate prior sensitization in vivo to antigens on this target cell.

3) In contrast to chronic hepatitis, spontaneous cell-mediated cytotoxicity (SCMC) of lymphocytes is defective in PBC. There is no defect in mitogen-induced cellular cytotoxicity (MICC) or antibody-dependent cellular cytotoxicity (ADCC) in either disease. In both diseases SCMC and ADCC are mediated by a subpopulation of lymphocytes which lack surface immunoglobulin and bear Fc receptors whereas MICC is mediated by lymphocytes which lack both surface immunoglobulin and Fc receptors. Thus, there is a difference in cytotoxic function in PBC and chronic hepatitis in that the former but not the latter disease is associated with a defect in the spontaneous cytotoxic function of lymphocytes which lack surface immunoglobulin but bear Fc receptors.

4) Starting with peripheral blood mononuclear cells, depletion of complement receptor-bearing cells is associated with enrichment of Fc positive and surface immunoglobulin negative cells, depletion of phagocytes and surface immunoglobulin positive cells and normal or enhanced SCMC, MICC and ADCC. In contrast, depletion of Fc positive cells is associated with enrichment of complement receptor-bearing cells and virtually no capacity to induce SCMC or ADCC. These findings indicate that at least the majority of cells which mediate SCMC, MICC and ADCC do not bear complement receptors.

5) The clearance of both tracer and test doses of microaggregated albumin is normal in patients with compensated PBC, alcoholic cirrhosis and HBsAg negative chronic hepatitis, indicating normal hepatic blood flow and a normal non-specific phagocytic capacity of the reticuloendothelial system.

The rate of clearance of IgG-coated radiochromated autologous red cells is normal in patients with compensated HBsAg negative chronic hepatitis and alcoholic cirrhosis. However, in the majority of patients with PBC, the rate of clearance of these cells was increased. This observation may imply activation of the reticuloendothelial system in this disease. In contrast, the clearance of C3b-coated radiochromated autologous red cells is markedly decreased in patients with compensated PBC, but is normal in patients with compensated HBsAg negative chronic hepatitis, alcoholic cirrhosis and large duct biliary obstruction. To obtain further insight into this clearance defect in PBC, a compartmental model of the C3b clearance process has been developed. Using computer simulations the model is able to account for the observed data and showed that the major determinant of the defect is a decreased rate of deposition of C3b-coated cells on Kupffer cell receptors. The defect cannot be attributed to cholestasis, to deficiency of components of the classical complement pathway, or to increased or decreased activity of C3b-inactivator. However, the defect may be due to a decrease in the number or affinity of C3b-receptors or to partial saturation of these receptors by excess circulating C3b or immune complexes containing C3b.

6) Cultures containing unirradiated T cells and B cells from normal individuals show augmentation of immunoglobulin (Ig) synthesis at low T:B cell ratios (helper T cell effect) and inhibition of Ig synthesis at high T:B cell ratios (suppressor T cell effect). Cultures containing irradiated T cells from normal individuals show augmentation of Ig synthesis at all T:B cell ratios studied, indicating marked reduction of suppressor T cell function by irradiation. In contrast, cultures containing unirradiated T cells and autologous B cells from patients with PBC do not show inhibition of Ig synthesis at high T:B cell ratios, but instead show progressive increases in Ig synthesis with increasing T:B cell ratios up to the highest T:B cell ratio studied (10:1). Ig synthesis by cultures containing irradiated T cells and autologus B cells from patients with PBC is similar to that by corresponding cultures of cells from normal subjects. Allogeneic mixtures of cells from normal individuals and patients with PBC show that, while B cells from the majority of patients with PBC synthesize normal amounts of Ig in the presence of normal T cells, T cells from patients with PBC cause marked enhancement of Ig synthesis by normal B cells. Patients with other liver diseases characterized by chronic cholestasis, cirrhosis, or hepatocellular necrosis do not exhibit an analogous abnormality of Ig synthesis <u>in vitro</u> to that found in PBC. Thus, PBC is associated with a defect of immune regulation characterized by abnormally high Ig synthesis by cultures of autologous T and B cells at high T:B cell ratios. This defect may be due to a deficiency of suppressor T cell function.

Significance to Biomedical Research:

Eludication of the pathogenetic mechanisms which initiate and sustain the disease processes of chronic active hepatitis and primary biliary cirrhosis is required for better diagnostic testing and development of rational therapies. Defined initiating factors exist for those subgroups of chronic active hepatitis which are associated with HBsAg and which follow the ingestion of certain drugs, whereas initiating factors are unknown for the remainder of patients with chronic active hepatitis and for all patients with primary biliary

cirrhosis. The processes leading to sustained disease activity are unknown in both conditions. Current hypotheses favor immunologic mechanism(s). These hypotheses, in part, stem from the high prevalence of abnormal immunologic phenomena in both diseases. The empiric use of adrenal corticosteroids and other immunosuppressive therapies in these diseases appears to be, at least in part, a reflection of this view. To date, existing specific hypotheses lack experimental confirmation. In addition, it is not known whether certain immunologic abnormalities found in some subgroups of patients and not in others are epiphenomena or are related to pathogenesis.

The current studies are designed to provide a comprehensive immunologic evaluation of selected patients so that the spectrum of immunologic abnormalities associated with primary biliary cirrhosis and HBsAg negative chronic hepatitis can be defined and the relationship of these abnormalities to clinical and histologic variables can be assessed. In addition, hypotheses of specific pathogenic mechanisms are tested. The significant morbidity and mortality of chronic active hepatitis, the uniform mortality of primary biliary cirrhosis, and the inadequacies of all current therapies in both conditions emphasize the need for information which throws light on the pathogenesis of these diseases.

Proposed Course:

The protocol, as described above, will be continued. Future investigations might be directed toward developing methods for the isolation of pure preparations of hepatocytes from animal livers and from biopsies of human livers for use as target cells in cytotoxicity assays. After adequate safeguards are instituted, patients with HBsAg positive chronic active hepatitis will be included within the protocol.

Publications:

1. Jaffe, C.J., Vierling, J.M., Jones, E.A., Lawley, T.J., and Frank, M.M.: Receptor specific clearance by the reticuloendothelial system in chronic liver diseases. Demonstration of defective C3b-specific clearance in primary biliary cirrhosis. J. Clin. Invest. 62: 1069-1077, 1978.

2. Potter, B.J., Elias, E., Fayers, P.M., and Jones, E.A.: Profiles of serum complement in patients with hepatobiliary diseases. Digestion 18: 371-383, 1978.

3. Jones, E.A., Frank, M.M., Jaffe, C.J., and Vierling, J.M.: Primary biliary cirrhosis and the complement system. Ann. Int. Med. 90: 72-84, 1979.

4. Jones, E.A., Vierling, J.M., Steer, C.J., and Reichen, J.: Cell surface receptors in the liver. In Popper, H. and Schaffner, F. (Eds.): Progress in Liver Diseases, Vol. VI. New York, Grune & Stratton, 1979, pp. 43-80.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,504-05 DDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies of Copper Metabolism in Man Using ^{67}Cu and ^{64}Cu

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Sec. Dis. of Liver	DDB:NIAMDD
Other:	R. Aamodt	Senior Radiobiologist	NM:CC
	W. Rumble	Biologist/Radiobiologist	NM:CC
	R. Shrager	Mathematician	LSMM:CR

COOPERATING UNITS (if any)
Nuclear Medicine Department, CC
Laboratory of Statistical and Mathematical Methodology, CR

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/4	1/4	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To study copper metabolism in vivo the fate of ^{67}Cu is followed after its intravenous injection or the fate of both ^{67}Cu and ^{64}Cu are followed after giving one of the isotopes intravenously and the other by mouth. Subsequently, measurements are made of radioactivity in the whole body, liver, red cells, ceruloplasmin, ceruloplasmin-free plasma, urine and feces. The data obtained are being analyzed by computer using curve-fitting, deconvolution and integration techniques. The computer analysis enables the kinetics of intestinal absorption, hepatic uptake, biliary excretion and incorporation into ceruloplasmin to be quantitated. The primary goal of these studies is to obtain an improved understanding of copper metabolism in normal subjects and in patients with diseases associated with increased deposition of copper in the liver, notably Wilson's disease and primary biliary cirrhosis. Another goal is to determine whether a set of abnormalities of radiocopper kinetics can be defined which would enable subjects who are heterozygous for Wilson's disease to be distinguished from those who are homozygous and those who are neither homozygous nor heterozygous for this disease.

Project Description:

Objectives:

To evaluate quantitatively the metabolism of copper in health and specific diseases associated with abnormalities of copper metabolism.

The ultimate goals include a comprehensive understanding of normal copper metabolism, and an evaluation of the significance of specific abnormalities of copper metabolism in patients who have increased deposition of copper in the liver, in particular those with hepatolenticular degeneration (Wilson's disease) and with diseases associated with chronic cholestasis such as primary biliary cirrhosis (PBC). In addition, relatives of patients with hepatolenticular degeneration, some of whom are heterozygotes for this condition, are also studied, to determine whether abnormalities of copper metabolism characteristic of the heterozygote state can be defined.

Methods Employed:

1) Studies using ^{67}Cu alone. After the intravenous injection of ^{67}Cu serial measurements are made of whole body radioactivity (using a whole body counter), hepatic radioactivity (using a scintillation probe), and radioactivity in red cells, ceruloplasmin-free plasma, ceruloplasmin, urine and feces. With the exception of hepatic radioactivity, these measurements are made for a period of 13 days. Serum ceruloplasmin-bound ^{67}Cu determination involves the removal of non-ceruloplasmin-bound ^{67}Cu from serum samples by addition of a copper chelating agent and passage of samples down small columns of activated charcoal. Plasma nonceruloplasmin-bound ^{67}Cu is determined by subtracting radioactivity in ceruloplasmin from that in whole plasma. The curves of total body, hepatic, red cell, plasma ceruloplasmin and plasma nonceruloplasmin radioactivity are defined and the total excretion of radioactivity in urine and feces calculated.

2) Studies using ^{67}Cu and ^{64}Cu. Studies are also being conducted in which the fate of simultaneously administered ^{67}Cu and ^{64}Cu is followed. One of the isotopes is given orally and the other intravenously. The subsequent protocol is the same as for studies using ^{67}Cu alone except that both ^{67}Cu and ^{64}Cu radioactivity are measured in all experimental specimens. In measuring ^{67}Cu and ^{64}Cu simultaneously an appropriate correction is made for the radioactivity due to ^{64}Cu which cannot be excluded when measuring ^{67}Cu. The data obtained are being analyzed by computer. This enables various parameters of copper metabolism to be quantitated, in particular the kinetics of intestinal absorption, hepatic uptake, biliary excretion and incorporation into ceruloplasmin.

Major Findings:

1) The ranges of whole body ^{67}Cu retention for normal subjects and homozygotes and heterozygotes for hepatolenticular degeneration have been defined.

2) Appreciable incorporation of ^{67}Cu into ceruloplasmin in normal subjects and patients with PBC has been demonstrated. In contrast, incorporation of ^{67}Cu into ceruloplasmin in homozygotes for hepatolenticular degeneration was minimal. Thus, incorporation of radiocopper into ceruloplasmin can be used to differentiate between two hepatic copper overload states, Wilson's disease and PBC.

3) A pool of nonceruloplasmin-bound copper which rapidly exchanges with plasma nonceruloplasmin-bound copper exists in normal subjects but is either saturated or absent in patients with PBC.

4) About 50% of orally administered radiocopper is absorbed in both normal subjects and patients with PBC.

5) In the presence of a positive copper balance in PBC, the degree of intestinal absorption and hepatic extraction of orally-administered radiocopper is inappropriate.

6) A major defect in copper metabolism in PBC is decreased biliary excretion of copper.

7) There is a decreased fractional rate of uptake of copper by the liver in Wilson's disease which is not attributable solely to an increased hepatic copper concentration.

8) The kinetics of incorporation of ^{67}Cu into ceruloplasmin in both normal subjects and patients with hepatic copper overload due to PBC suggest that initially there is rapid equilibration of ^{67}Cu with a large hepatic pool of copper which has a slow turnover rate and a loss pathway with a small fractional transfer rate to the precursor pool of copper for ceruloplasmin synthesis. These kinetics also suggest that there is a rapid pathway for the incorporation of radiocopper into ceruloplasmin which bypasses the hepatic storage pool. Incorporation of ^{67}Cu into ceruloplasmin via the rapid pathway is significantly reduced in PBC.

Significance to Biomedical Research

In certain disease states marked variations in the copper content of plasma and various organs are known to occur. However, the mechanisms by which copper homeostasis is maintained in health and disturbed in disease are poorly understood. Increasing attention has been focused on copper metabolism in hepatolenticular degeneration since the refutation of the concept that a low ceruloplasmin concentration is primarily responsible for manifestations of the disease. In hepatolenticular degeneration and in diseases which are associated with chronic cholestasis, such as PBC, chronic large duct biliary obstruction, progressive intrahepatic cholestasis of infancy and childhood and sclerosing cholangitis, very high concentrations of copper have been demonstrated in the liver. It has been suggested that increased hepatic copper may contribute to tissue injury and that reduction of hepatic copper by copper chelation therapy may improve the prognosis in these diseases. By the use of the double radionuclide technique outlined it is possible to determine whether specific disorders characterized by increased hepatic deposition of

copper are associated with particular defects in copper metabolism, such as abnormalities of intestinal absorption, hepatic uptake, biliary secretion and incorporation into ceruloplasmin. The pathogenesis of neither hepatolenticular degeneration nor primary biliary cirrhosis is known, and defects in hepatic handling of copper in these diseases could be either directly related to their pathogenesis or secondary phenomena potentiating tissue injury.

By studying heterozygotes for hepatolenticular degeneration, the effect of the presence of a single gene for this condition on copper metabolism can be defined.

Proposed Course:

Data from the studies are being analyzed. No further studies are planned at this time.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,505-04 DDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies of the Metabolism of Radioiodinated Alpha-1-Antitrypsin and Desialylated Alpha-1-Antitrypsin in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Sec. Dis. of Liver	DDB:NIAMDD
Other:	J. Vergalla	Chemist	DDB:NIAMDD
	C. J. Steer	Clinical Associate	DDB:NIAMDD
	J. Karsh	Guest Worker	A&R:NIAMDD
	J. E. Gadek	Clinical Associate	CB:NHLBI
	R. G. Crystal	Head, Pulmonary Branch	PB:NHLBI

COOPERATING UNITS (if any)
Pulmonary Branch, NHLBI

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/4	1/4	1

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Highly purified biologically active alpha-1-antitrypsins (αlAT's) are being isolated from plasma using gel exclusion chromatography, affinity chromatography, and DEAE ion exchange chromatography. Isolated preparations of αlAT, trace-labeled with radioiodine, are being used to obtain data on the turnover of αlAT in normal subjects, patients with αlAT deficiency and their relatives and patients with chronic hepatocellular disease. The protease inhibitor phenotypes of subjects donating plasma for isolation of αlAT and subjects undergoing αlAT turnover studies are determined. αlAT's, isolated from normal subjects (phenotype PiMM) and from patients with αlAT deficiency (phenotype PiZZ) have different degrees of sialylation. Each is labeled with a different isotope of iodine and the metabolism of the two labeled preparations are compared in the same individuals. In addition, the metabolism in vivo of αlAT which has been desialylated in vitro is being studied. The relationship between the degree of desialylation of αlAT and its rate of catabolism and the extent to which decreased hepatic release or increased hepatic uptake of αlAT contributes to the low plasma concentrations of αlAT in αlAT deficiency are being determined.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Alpha-1-antitrypsin (α1AT) is a plasma glycoprotein which contains sialic acid and is responsible for most of the trypsin inhibitory capacity of plasma. The objectives of this study are to obtain data on the metabolism of α1AT in normal subjects (phenotype PiMM), patients with α1AT deficiency (phenotype PiZZ), relatives of PiZZ individuals with one Z gene and patients with hepatocellular disease unassociated with α1AT deficiency. The possibility that the degree of sialylation of this protein, which is less in PiZZ than PiMM individuals, influences its metabolism in vivo will be investigated by comparing the metabolism of α1AT's isolated from PiMM and PiZZ individuals in the same subjects and by studying the metabolism of α1AT which has been desialylated in vitro. The necessity to perform protease inhibitor (Pi) phenotyping for these studies has permitted additional studies in which the distribution of phenotypes in selected populations has been determined.

Methods Employed:

1) The Pi phenotypes of subjects donating plasma for isolation of α1AT and subjects undergoing studies of α1AT metabolism are determined using acid starch gel electrophoresis followed by crossed immunoelectrophoresis and/or slab gel isoelectric focusing in acrylamide.

2) α1AT is isolated from plasma using $(NH_4)_2SO_4$ precipitation, gel exclusion chromatography, Concanavalin A affinity chromatography, DEAE ion exchange chromatography and affinity chromatography using blue dextran and activated thiol exchange.

3) The purity of isolated α1AT is determined using crossed immunoelectrophoresis, polyacrylamide gel electrophoresis and isoelectric focusing. Its biological activity is assessed by a kinetic assay of tryptic inhibitory capacity using an artificial substrate.

4) To quantitate the turnover of α1AT in man, pure biologically active α1AT trace labeled with radioiodine is administered intravenously and serial measurements of radioactivity in the whole body, plasma and urine are analyzed.

Major Findings:

1) Isolated (PiMM) α1AT has been shown to be immunochemically pure and to retain both its characteristic electrophoretic microheterogeneity on acid starch gel electrophoresis and its ability to inhibit trypsin.

2) The half life of radioiodinated human (PiMM) α1AT in the plasma of rabbits varied from 8.7 to 10.4 hours whereas that of the same protein after desialylation was less than 10 minutes.

3) Ranges for pool sizes, transfer rates between pools and synthetic and fractional catabolic rates of α1AT in normal subjects have been defined.

4) In turnover studies of MM αlAT in normal (PiMM) volunteers, the serum concentration of αlAT was shown to vary independently of its fractional catabolic rate suggesting that a constant volume of plasma is cleared of α_1AT per unit time. Analysis of the turnover data indicated that α_1AT is catabolized in, or in close relationship to, the intravascular compartment.

5) In analogous studies in normal volunteers the desialylated protein was shown to be removed from plasma approximately 150 times faster than the intact protein. Its rapid disappearance from plasma was associated with an equally rapid uptake of the radioiodine label by the liver. This finding emphasizes that the catabolism of αlAT can be influenced by the molecular structure of the carbohydrate moiety and suggests the existence of hepatic receptors for desialylated glycoproteins in the human liver.

6) A patient with the rare Pi phenotype SZ, αlAT deficiency and cirrhosis with globules of αlAT material in his hepatocytes was shown to have appreciable hepatic copper overload, chronic fibrosing interstitial pneumonitis and, terminally, a hepatocellular carcinoma.

7) No significant increase in non-MM Pi phenotypes was found in 37 patients with rheumatoid arthritis and 40 patients with systemic lupus erythematosus suggesting that particular Pi types neither predispose to the development nor enhance the severity of these diseases.

8) No significant increase in non-MM Pi phenotypes was found in 52 patients with Sjogrens syndrome. Mean values for tests of pulmonary function, including estimates of both restrictive lung disease and airway obstruction were not significantly different for patients with Sjogren's syndrome who had the Pi MM phenotype and those with non-MM Pi phenotypes.

Significance to Biomedical Research:

Homozygous αlAT deficiency (phenotype PiZZ) may be associated with cholestasis in the neonatal period, cirrhosis, liver cell carcinoma and emphysema at a young age. In this condition, hepatocytes have been shown to contain globules of αlAT-like material which is devoid of sialic acid, and the αlAT in plasma, which is present in abnormally low concentration, has also been shown to have a reduced content of sialic acid. The relationship of the characteristic hepatic globules and the low plasma concentration of αlAT to the hepatic lesions associated with αlAT deficiency is unknown. This study is designed to investigate the metabolism of αlAT in αlAT deficiency. The relationship of the characteristic hepatic globules to the rate at which αlAT is delivered to plasma and to the concentration of αlAT in plasma will be assessed. In addition, the contribution of increased catabolism or hepatic uptake of αlAT to reduced plasma concentrations of αlAT will be determined. The study will establish the effect of variations in the molecular structure of αlAT (e.g. different degrees of sialylation) on its rate of endogenous catabolism or hepatic uptake. In particular, the hypothesis that there are hepatic receptors for exposed galactosyl residues on desialylated glycoproteins which facilitate the rapid hepatic clearance of these proteins will be tested

in man. The effect of chronic hepatocellular disease on the liver's capacity to clear a desialylated glycoprotein will be determined. In addition, the influence of chronic hepatocellular disease per se on the metabolism of αlAT will be studied so that important control data can be generated for the evaluation of αlAT metabolism in patients with αlAT deficiency associated with chronic hepatocellular disease. The observation that desialylated glycoproteins appear to be metabolized by hepatic lysosomes suggests that measurements of hepatic radioactivity after administering a radioiodinated desialylated glycoprotein such as αlAT could be used as a test for hepatic lysosomal function.

Proposed Course:

It is proposed to administer labeled intact and desialylated (PiMM) αlAT to patients with αlAT deficiency (phenotype PiZZ) and to administer labeled αlAT isolated from patients with αlAT deficiency (phenotype PiZZ), who do not have associated hepatocellular disease, to both normal subjects (phenotype PiMM) and patients with αlAT deficiency (phenotype PiZZ). These studies will enable the contribution of decreased hepatic release of αlAT and increased hepatic uptake of αlAT to the low serum concentration of αlAT in αlAT deficiency to be assessed. Whether αlAT that is biosynthetically deficient in sialic acid behaves differently from that which is deficient in sialic acid as a result of neuraminidase treatment will also be determined by these studies.

Publications:

1. Jones, E.A., Vergalla, J., Steer, C.J., Bradley-Moore, P.R., and Vierling, J.M.: Metabolism of intact and desialylated α_1-antitrypsin. Clin. Sci. Molec. Med. 55: 139-148, 1978.

2. Chan, C.H., Steer, C.J., Vergalla, J., and Jones, E.A.: Alpha-1-antitrypsin deficiency with cirrhosis associated with the protease inhibitor phenotype SZ. Amer. J. Med. 65: 978-985, 1978.

3. Karsh, J., Vergalla, J, and Jones, E.A.: Alpha-1-antitrypsin phenotypes in rheumatoid arthritis and systemic lupus erythematosus. Arth. Rheum. 22: 111-113, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 53,506-03 DDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies of the Properties of Desialylated Human Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.:	E. A. Jones	Acting Chief, Sec. Dis. of Liver	DDB:NIAMDD
Other:	C. J. Steer	Clinical Associate	DDB:NIAMDD
	S. P. James	Clinical Associate	DDB:NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/4	1/4

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
A glycoprotein isolated from rabbit hepatocytes (HBP) which has the ability to bind specifically desialylated glycoproteins is also capable of inducing mitogenesis in human desialylated lymphocytes. The immunogenic properties of HBP, the first lectin of mammalian origin to be recognized, is being studied further. It has been shown that isolated HBP has the ability to increase the cytotoxicity of human desialylated lymphocytes against Chang target cells to the same degree as the plant lectin phytohemagglutinin. HBP did not increase the cytotoxicity of intact lymphocytes. In addition, it has been shown that desialylated lymphocytes induced significantly more cytotoxicity in isolated rabbit hepatocytes, which bear HBP on their surface, than did intact lymphocytes, and that this phenomenon could be blocked by asialo-orosomucoid (a desialylated glycoprotein) but not by intact orosomucoid. These findings suggest that the cytotoxicity induced by membrane-bound HBP is similar in mechanism to that induced by the isolated protein and that the induced cytotoxicity probably depends on the interaction between HBP and exposed galactosyl residues on desialylated lymphocytes. Desialylated lymphocytes are selectively taken up by the liver. Studies to determine the mechanism responsible for this process have been undertaken.

Project Description:

Objectives:

HBP is a normal constituent of the surface membranes of mammalian hepatocytes which can induce mitogenesis in desialylated lymphocytes. This project is designed to determine whether HBP has additional immunologic properties such as the ability to induce desialylated lymphocytes to become cytotoxic, that is to mediate mitogen-induced cellular cytotoxicity (MICC). Desialylated lymphocytes are selectively taken up by the liver. Studies are designed to determine the mechanism of this phenomenon, including the role, if any, of HBP.

Methods Employed:

HBP is isolated in a pure form from rabbit liver using affinity chromatography. HBP in the surface membranes of hepatocytes is measured by its ability to bind ^{125}I-asialo-orosomucoid. For assays of cytotoxicity lymphocytes are isolated from the blood of normal human volunteers by centrifugation over Ficoll-Hypaque. Lymphocytes are desialylated by incubating with neuraminidase. Two in vitro assays of cytotoxicity are used. One measures the release of radioactivity from ^{51}Cr-labeled nucleated target cells after their incubation with effector cells in the presence or absence of mitogens. The other measures the loss of ability of target cells to adhere to plastic after incubation with effector cells. Viability of both effector and target cells is measured by the ability of cells to exclude the dye trypan blue. Chang cells are used as nonspecific nucleated target cells. Pure rabbit hepatocytes, isolated by perfusing the liver in situ with a solution containing collagenase and hyaluronidase, are used as hepatospecific target cells containing exposed HBP on their surface membranes. The hepatic uptake of ^{51}Cr-labeled desialylated and normal lymphocytes and the effects of complement depletion in vivo with cobra venom factor and preincubation of the labeled desialylated cells with rabbit IgG Fab fragments on the hepatic uptake of desialylated lymphocytes are determined from measurements of radioactivity in plasma, liver and other organs in rabbit.

Major Findings:

1. Cytotoxicity of desialylated lymphocytes against nonspecific nucleated target cells in the presence of HBP was equivalent to that in the presence of the powerful plant mitogen phytohemagglutinin and was much greater than that induced by these cells in the absence of HBP. After prolonged culturing of desialylated lymphocytes in the presence of HBP, this protein was found to induce blast transformation and the progeny cells exhibited increased cytotoxicity compared to desialylated lymphocytes cultured in the absence of HBP. Having shown that isolated rabbit hepatocytes contained exposed HBP in their surface membrane, it was found that a much greater proportion of these cells lost their ability to adhere to plastic after incubation with desialylated lymphocytes than after incubation with normal untreated lymphocytes. This effect of desialylated lymphocytes was specifically inhibited by asialo-orosomucoid, a desialylated glycoprotein, but not by intact orosomucoid. These findings indicate that HBP can mediate MICC not only as a

purified protein but also as a constituent of the surface membrane of a mammalian hepatocyte.

2. The selective hepatic uptake of the label of ^{51}Cr-labeled desialylated peripheral blood mononuclear cells has been demonstrated in the rabbit. This phenonenon does not appear to be due to the specific interaction between exposed galactosyl residues on the surface of the desialylated cells and the hepatic binding protein which specifically recognizes exposed galactosyl residues on desialylated glycoproteins, since sinusoidal cells, which come into contact with circulating desialylated cells do not bind desialylated glycoproteins and hence do not bear hepatic binding protein. Consistent with this reasoning is the observation that after infusions of desialylated lymphocytes into rabbits neither serial values for serum transaminases nor hepatic histology were indicative of hepatocellular injury. Naturally occurring antibodies exist in normal plasma which specifically recognize antigens exposed on the surface of lymphocytes by desialylation. These antibodies probably do not mediate the selective hepatic uptake of desialylated cells through the action of complement as complement depletion of rabbits in vivo was without effect on the hepatic uptake of the label of ^{51}Cr-labeled desialylated cells. However, preincubation of labeled desialylated peripheral blood mononuclear cells with rabbit IgG Fab fragments reduced the hepatic uptake of the label of these cells to that of the label of ^{51}Cr-labeled intact peripheral blood mononuclear cells suggesting that the Fab fragments were able to bind to exposed antigens on desialylated cells and inhibit binding of circulating antibodies to these antigens. These findings are compatible with the hypothesis that desialylated lymphocytes in vivo become coated with naturally occurring antibodies with subsequent recognition of cell-bound antibody by Fc receptors on Kupffer cells mediating their selective hepatic uptake.

Significance to Biomedical Research:

For the first time MICC has been shown to be mediated by a normal constituent of the surface membrane of a mammalian cell. The results of these studies demonstrate that a minor structural change in the surface membrane of lymphocytes might result in their becoming cytopathogenic when exposed to normal hepatocytes, suggesting that MICC may be a mechanism of hepatocellular injury. This observation raises the possibility that interaction between a variety of constituents of normal cell membranes and lymphocytes whose surface membranes have undergone subtle structural changes may be a mechanism of immune-mediated cellular injury in human disease. The clearance of desialylated lymphocytes by Fc receptors on Kupffer cells in vivo may prevent the mediation of hepatocellular injury by the interaction of desialylated lymphocytes with HBP on the surface of hepatocytes. These observations may be particularly significant since the enzyme responsible for desialylation, neuraminidase, appears to be widely distributed in vivo and occurs in organisms pathogenic to man such as the influenza virus.

Proposed Course:

It is proposed to terminate this project when the studies described are completed.

Publications:

1. Vierling, J.M., Steer, C.J., Hickman, J.W., James, S.P., and Jones, E.A.: Cell mediated cytotoxicity of desialylated human lymphocytes induced by a mitogenic mammalian liver protein. Gastroenterology 75: 456-461, 1978.

2. Jones, E.A., Vierling, J.M., Steer, C.J., and Reichen, J.: Cell surface receptors in the liver. In Popper, H. and Schaffner, F. (Eds.): Progress in Liver Diseases, Vol. VI. New York, Grune & Stratton, 1979, pp. 43-80.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,507-02 DDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Synthesis and Binding of Collagen by Hepatocytes in Chronic Liver Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	M. D. Berman	Clinical Associate	DDB:NIAMDD
Other:	J. G. Waggoner	Chemist	DDB:NIAMDD
	H. K. Kleinman	Senior Staff Fellow	LDBA:NIDR
	J. M. Foidart	Fogarty Fellow	LDBA:NIDR

COOPERATING UNITS (if any)
Laboratory of Developmental Biology and Anomalies, NIDR

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/4	1/4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interaction between hepatocytes and collagen may be of importance in the pathogenesis of chronic hepatocellular diseases. The adherence of each of the five types of collagen to hepatocytes isolated from normal rats and rats with carbon tetrachloride-induced hepatic fibrosis has been studied. Attachment of hepatocytes from normal rats was enchanced four-fold by the presence of either serum or fibronectin, the glycoprotein required for fibroblast attachment to collagen. In the absence of serum or fibronectin, hepatocytes adhered best to basement membrane collagen (type IV). This phenomenon was abolished by sodium periodate oxidation of collagen, suggesting that hepatocytes were attached to collagen by recognizing a specific carbohydrate residue. Hepatocytes isolated from rats with hepatic fibrosis bound less avidly to collagen than those from normal rats. Neither hepatocytes from normal rats nor those from rats with hepatic fibrosis appear to synthesize collagen in vitro. These studies indicate a defect in the interaction of hepatocytes with collagen in an animal model of chronic liver disease.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To determine whether the interaction between hepatocytes and collagen may be abnormal in chronic hepatocellular disease. The ultimate objectives of this project is to define the factors responsible for the laying down of increased quantities of collagen in the liver in chronic liver diseases and to determine how the progression of hepatic fibrosis might be inhibited or prevented.

Methods Employed:

1) The induction of hepatic fibrosis in rats using carbon tetrachloride.

2) The preparation of suspensions of cells from rat livers perfused with a solution containing collagenase and the subsequent isolation of pure preparations of hepatocytes by density sedimentation.

3) Cultures of hepatocytes with different types of collagen.

4) Immunofluorescent staining of cells with antibodies specific for different procollagens.

5) Measurements of the incorporation of the label of ^{3}H-proline into proteins synthesized by hepatocytes.

Major Findings:

1) Attachment of hepatocytes from normal rats to collagen was enhanced four-fold by the presence of serum or fibronectin, the glycoprotein required for fibroblast attachment to collagen.

2) In the absence of fibronectin hepatocytes adhered best to basement membrane collagen (type IV).

3) Periodate oxidation of collagen abolishes its binding to hepatocytes suggesting that the carbohydrate moiety of collagen may be involved in the binding.

4) Hepatocytes isolated from rats with hepatic fibrosis bind to collagen less avidly that do those from normal rats.

5) Procollagens were not demonstrated immunofluorescently in hepatocytes but were found in cells with fibroblastoid morphology.

6) Hepatocytes from normal rats and from rats with hepatic fibrosis do not appear to synthesize collagen.

Significance to Biomedical Research:

Hepatic fibrosis is a major component of the disturbed hepatic architecture in most chronic liver diseases. In cirrhosis it is associated with the development of portal hypertension and episodes of bleeding into the gastrointestinal tract from esophageal varices. This project is designed to investigate the pathogenesis of the abnormal laying down of collagen and fibrosis in the liver in chronic liver disease. The results obtained may suggest means of decreasing the rate of progression of hepatic fibrosis or preventing it altogether.

Proposed Course:

Data from recent studies are being analyzed. No further studies are being planned at this time.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,508-02 DDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies of Hepatic Receptors for Modified Glycoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. A. Jones	Acting Chief, Sec. Dis. of Liver	DDB:NIAMDD
Other:	C. J. Steer	Clinical Associate	DDB:NIAMDD
	S. Furbish	Senior Staff Fellow	DMNB:NINCDS
	J. A. Barranger	Chief, Clinical Section	DMNB:NINCDS
	R. O. Brady	Chief, Dev. Met. Neur. Br.	DMNB:NINCDS
	R. Clarenberg	Guest Worker	LBM:NIAMDD

COOPERATING UNITS (if any)
Laboratory of Biochemistry and Metabolism
Development and Metabolic Neurology Branch, NINCDS

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The binding of radiolabeled modified glycoproteins to parenchymal and non-parenchymal cell preparations isolated from the perfused rat liver has been studied. Hepatocytes possess receptors for glycoproteins with galactose as the terminal sugar residue. Non-parenchymal cells lack a receptor for galactose-terminated glycoproteins but specifically bind N-acetylglucosamine and mannose-terminated glycoproteins. The effect of modifying the carbohydrate moiety of glucocerebrosidase on the distribution of its cellular uptake after intravenous infusion into rats was studied. The carbohydrate moiety of this enzyme could be modified without loss of enzyme activity. Galactose-terminated glucocerebrosidase was selectively taken up by hepatocytes. Exposure of N-acetylglucosamine or mannose residues markedly augmented the uptake of the enzyme by Kupffer cells. The ability to manipulate the cellular distribution of an infused enzyme may be of importance in enzyme replacement therapy. The intact glycoprotein, human ß-hexosaminidase A is rapidly cleared from the circulation by a mannose-specific recognition system on hepatic sinusoidal cells. This process may be of physiologic importance in preventing the accumulation of potentially autodestructive lysosomal enzymes in the circulation.

Project Description:

Objectives:

This study was designed to determine whether receptors for modified glycoproteins, other than that previously described for galactose-terminated glycoproteins (HBP), exist in the mammalian liver. It was also designed to assess the distribution of receptors for modified glycoproteins, including HBP, between parenchymal and non-parenchymal hepatic cells. The potential implications of these receptors to clinical medicine has been the subject of additional studies. In particular, the effects of modifying the carbohydrate moiety of glucocerebrosidase, the enzyme deficient in Gaucher's disease, on its cellular distribution after infusion has been determined. The cell type responsible for the rapid hepatic clearance of a lysosomal enzyme (β-hexosamindase A) and the glycosyl specificity of this process were investigated.

Methods Employed:

1) Suspensions of hepatic cells are obtained from rat liver after in situ perfusion with a solution of collagnease. Parenchymal and non-parenchymal hepatic cells are subsequently isolated from cell suspensions using gravity sedimentation and Metrizamide density gradient centrifugation.

2) For binding studies, the carbohydrate moiety of native glycoproteins are modified by incubations with appropriate enzymes (neuraminidase, β-galactosidase, N-acetylglucosaminidase).

3) Glucocerebrosidase activity is assayed in detergent buffered extracts of sonicated cells.

4) Glycoproteins are radioiodinated using Enzymobeads and the Chloramine-T method.

5) Standard assay techniques are employed to determine the uptake of labeled ligands by cells incubated in vitro.

Major Findings:

1) Significant binding of galactose, but neither N-acetylglucosamine nor mannose-terminated orosomucoid to intact hepatocytes was demonstrated. Non-parenchymal cells did not bind galactose-terminated orosomucoid but bound significant amounts of N-acetylglucosamine and mannose-terminated orosomucoid. Thus, Kupffer cells lack a receptor for galactose-terminated glycoproteins. Carrier dilution studies indicated the presence of a distinct receptor for galactosyl-terminated glycoproteins on hepatocytes and a distinct receptor with mannosyl specificity on non-parenchymal cells.

2) The carbohydrate moiety of the enzyme glucocerebrosidase can be modified without the glycoprotein losing enzyme activity. Galactose-terminated glucocerebrosidase is preferentially taken up by hepatocytes.

Exposure of N-acetylglucosamine or mannose sugar residues markedly augments the uptake of glucocerebrosidase by non-parenchymal hepatic cells.

3) The rapid in vivo hepatic uptake of the glycoprotein β-hexosaminidase A exhibits mannosyl specificity. In vitro no uptake of this enzyme by hepatocytes is detectable, whereas there is appreciable uptake of the enzyme by sinusoidal cells. This uptake process also exhibits mannosyl specificity. The kinetics of uptake of β-hexosaminidase A and mannose-terminated orosomucoid by sinusoidal cells are similar.

Significance to Biomedical Research:

Hepatic receptors for glycoproteins with different terminal sugar residues may be of importance in the catabolism of glycoproteins and in uptake of cells with modified glycoproteins on their surface membrane. The ability to manipulate the cellular distribution of an infused glycoprotein enzyme by modifying its carbohydrate moiety may be of major clinical importance in enzyme replacement therapy. For instance, in Gaucher's disease, in which there is a deficiency of the enzyme glucocerebrosidase, glucocerebroside accumulates in Kupffer cells. Increased uptake of infused glucocerebrosidase by Kupffer cells may be achieved by exposing mannose as the terminal sugar of the carbohydrate moiety. Receptor mediated hepatic clearance of lysosomal enzymes, such as β-hexosaminidase A, may be of physiologic importance in preventing the accumulation in the circulation of potentially autodestructive enzymes.

Proposed Course:

Studies are in progress to determine the number of galactose-specific receptor sites on the hepatocyte, as well as to define the kinetics of uptake of galactose-terminated glycoproteins by hepatocytes.

Publications:

1. Steer, C.J., Furbish, F.S., Barranger, J.A., Brady, R.O., and Jones, E.A.: The uptake of agalacto-glucocerebrosidase by rat hepatocytes and Kupffer cells. FEBS Letters 91: 202-205, 1978.

2. Barranger, J.A., Penchev, P.G., Furbish, F.S., Steer, C.J., Jones, E.A., and Brady, R.O.: Studies of lysosomal function. I. Metabolism of some complex lipids by isolated hepatocytes and Kupffer cells. Biochem. Biophys. Res. Commun. 83: 1055-1060, 1978.

3. Jones, E.A., Vierling, J.M., Steer, C.J., and Reichen, J.: Cell surface receptors in the liver. In Popper, H. and Schaffner, F. (Eds.): Progress in Liver Diseases, Vol. VI. New York, Grune & Stratton, 1979, pp. 43-80.

4. Steer, C.J., and Clarenberg, R.: Unique distribution of glycoprotein receptors on parenchymal and sinusiodal cells of rat liver. J. Biol. Chem. 254: 4457-4461, 1979.

5. Steer, C.J., Kusiak, J.W., Brady, R.O., and Jones, E.A.: Selective hepatic uptake of human β-hexosaminidase A by a specific glycoprotein recognition system on sinusoidal cells. Proc. Nat. Acad. Sci. (USA) 76: 2774-2778, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 53,509-01 DDB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies of patients who are chronic carriers of hepatitis B surface antigen (HBsAg)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	J. H. Hoofnagle	Senior Investigator	DDB:NIAMDD
Other:	E. A. Jones	Acting Chief, Sec. Dis. of Liver	DDB:NIAMDD
	S. P. James	Clinical Associate	DDB:NIAMDD
	D. F. Schafer	Clinical Associate	DDB:NIAMDD
	R. H. Purcell	Chief, Hepatitis Section	LID:NIAID
	S. M. Finestone	Senior Investigator	LID:NIAID
	J. Gerin	Head, Molec. Virol. & Immunol. Div.	Georgetown University

COOPERATING UNITS (if any)

LID, NIAID
Georgetown University School of Medicine

LAB/BRANCH
Digestive Diseases Branch

SECTION
Section on Diseases of the Liver

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/4	1/4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Persons with HBsAg-positive chronic hepatitis are being enrolled in a prospective study of this disease. Fifteen persons have been entered into this study, 12 of whom have been followed for at least 6 months, 9 of whom have had liver biopsies. At the start all 15 had elevated serum enzymes and were HBsAg positive; 12 had HBeAg and 11 DNA-polymerase. On follow-up, all have remained HBsAg positive but two have had a spontaneous fall in HBsAg titer and both have lost HBeAg. One of the two has become DNA-polymerase negative. The remaining patients have had stable levels of HBsAg, HBeAg and DNA-polymerase. In individuals with stable hepatitis B virus markers, trials of antiviral agents are being planned. A new drug - phosphonoformate - is currently being evaluated in HBsAg positive chimpanzees by Dr. Purcell's laboratory. Applications with the pharmaceutical company (Astra) have been made to start a trial of this drug in human chronic HBsAg carriers.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

This study was designed to establish a group of patients with well characterized chronic HBsAg-positive hepatitis in preparation for treatment trials with antiviral agents.

Methods Employed:

Assays have been established for detection and quantification of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) and DNA-polymerase. Assays for antibodies to HBsAg (anti-HBs) , antibody to hepatitis B core antigen (anti-HBc) and antibody to HBeAg (anti-HBe) and means of separating IgG from IgM class of antibodies have been established. Liver biopsies are being processed for light, immunofluorescence and electron microscopy.

Major Findings:

In the first six months of this project 15 HBsAg-positive persons have been enrolled and followed. All 15 persons are HBsAg positive with amounts of HBsAg ranging from 8-200 mcg/ml. In six months of follow-up, these levels have remained constant in 13 but have fallen in two. Twelve persons had HBsAg and eleven DNA-polymerase. In follow-up evaluation, two persons lost HBcAg and one of them DNA-polymerase. These were the same two who had a significant decrease in HBsAg titer. Liver biopsies have been performed on eight of the 15 (5 others had a recent biopsy at other institutions). Among 13 who have had recent liver biopsies, 8 have chronic active hepatitis, one has cirrhosis with chronic active hepatitis and four have chronic persistent hepatitis. There was no correlation of liver biopsy morphology and HBsAg titer, HBeAg or DNA-polymerase. All 15 had elevated serum enzymes indicative of chronic hepatitis and ranging from 1.5 times to 10 times normal. These enzyme levels have fluctuated widely in many of them. Both persons with a decrease in HBsAg and HBeAg have had a fall in serum enzymes towards normal serum transaminases (both are now < 1.5 times normal). Six of the fifteen persons have mild symptoms attributable to chronic hepatitis (malaise or arthralgias). None of the fifteen are jaundiced.

Significance to Biomedical Research:

The goal of this project is to investigate the natural history of chronic HBsAg positive hepatitis and investigate new therapies. At present there are approximately 1-2 million Americans who are chronic HBsAg carriers. Many of these have chronic liver disease and all are potentially infectious for their close contacts. There are no known treatments for the chronic HBsAg carrier state and little is known about its natural history. In these studies we hope to assess newly proposed therapies. For an adequate assessment, a group well characterized patients with stable hepatitis B virus markers is essential.

Proposed Course:

We plan to continue to follow the present 15 persons and continue to add

additional patients until we have 25-30 persons with stable markers of active hepatitis B virus infection (DNA-polymerase and HBeAg positive). Such persons will then be enrolled in treatment trials of antiviral agents. At present the Hepatitis Branch of the Laboratory of Infectious Diseases is screening antiviral agents in HBsAg-positive chimpanzees (the animal model of this disease). Promising antiviral agents will then be screened in these patients. At the present time a new antiviral agent, phosphonoformate, is being evaluated. It inhibits DNA-polymerase and preliminary data suggests that it causes a decrease in HBsAg titer. Application has been made with the manufacturers of phosphonoformate (Astra Pharmaceuticals) to file a joint IND with the Food and Drug Administration for a human trial using this drug intravenously.

Publications:

1. Hoofnagle, J.H.: Prevention of type B hepatitis. Gastroenterology 76: 1483-1494, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54007-05 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Effects of Short-term Fasting on Biliary Lipid Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P.H. Bennett, Acting Chief PCRS, NIAMDD

COOPERATING UNITS (if any)
L.J. Bennion, Sacramento, California (formerly Staff Physician,PCRS,NIAMDD)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Gallbladder bile lipid composition, bile acid composition, and bile acid pool sizes were measured in a group of 52 normal subjects studies under baseline conditions in this and several related projects were analyzed in relationship to sex, body size, and bile saturation.

This project has been completed and terminated.

Publications:

Bennion,L.J., Drobny,E., Knowler, W.C., Ginsberg, R.L., Garnick, M.B., Adler, R.D. and Duane, W.C.: Sex Differences in the Size of Bile Acid Pools. Metabolism 27:961-969,1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54012-05 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Effect of Dietary Fiber upon Biliary Lipid Composition

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P.H. Bennett, Acting Chief PCRS, NIAMDD
Other: None

COOPERATING UNITS (if any)
M. Hendrikx, Research Dietician, PCRS, IHS
M. Garnick, Sidney Farber Cancer Institute, Harvard Med. School, Boston, Mass.

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Populations ingesting a high fiber content in their diet are reputed to have a lower incidence of cholesterol cholelithiasis. Consequently, we measured gallbladder bile lipid composition and bile acid composition in 5 normal humans after ingestion of a low bran diet and again after ingestion of an otherwise identical high bran diet to determine the effects of dietary fiber on bile composition.

This project has been completed and terminated.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54013-05 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Effect of Oral Contraceptives on Glucose Tolerance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P. H. Bennett, Acting Chief PCRS, NIAMDD

Other: None

COOPERATING UNITS (if any)
S.L. Aronoff, Dept. of Medicine, Washington University, St. Louis, MO

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Oral contraceptives were previously shown to induce an abnormal cortisone glucose tolerance in subjects who had otherwise normal oral glucose tolerance tests. To investigate the mechanisms of this change, subjects planning to start or stop oral contraceptives were tested by means of an oral glucose tolerance test and cortisone glucose tolerance test with the measurement of glucose and insulin responses during each test, both before, during and after taking oral contraceptives.

The project has been completed and terminated.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54014-05 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Low Density Lipoprotein Metabolism in American Indians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barbara V. Howard	Research Biochemist	PCRS, NIAMDD
Other:	Peter H. Bennett	Acting Chief	PCRS, NIAMDD

COOPERATING UNITS (if any)
M. Garnick, Sidney Farber Cancer Institute, Harvard Medical School, Boston, MA
T. Langer, Dept. of Medicine, University of Pennsylvania

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Southwestern American Indians have lower serum cholesterol levels and lower prevalence of atherosclerosis than Caucasian control groups. To characterize these differences, we are investigating the lipoprotein cholesterol content and the kinetics of radiolabelled autologous low density lipoprotein in Southwestern American Indians and controls. The results of this study will elucidate the mechanisms responsible for the low LDL levels in this population and their relation to atherosclerosis.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Serum cholesterol levels are decreased and there is a low prevalence of atherosclerosis in southwestern American Indians compared to non-Indian populations. Since LDL is the main cholesterol bearing lipoprotein, LDL metabolism has been studied in order to determine if the low cholesterol levels are reflected in the differences in synthesis or metabolism.

Methods Employed:

1. Patients were stabilized on a low cholesterol, low saturated fat diet for three weeks.
2. LDL was isolated from plasma by ultracentrifugation.
3. LDL was iodinated with ^{125}I by the iodine monochloride method.
4. The preparation was tested for immunologic purity (agarose gel electrophoresis), pyrogens and sterility according to U.S.P. standards.
5. The radio-labelled preparation was injected intravenously; plasma decay curves and urinary excretion of radioactivity were measured daily to determine the T 1/2 and FCR, and LDL synthetic rate.

Major Findings: The absolute synthetic rate and total pool of apo-LDL were significantly lower in the Indians than Caucasians. In addition, concentrations apo-LDL and LDL cholesterol were significantly lower and the concentration of HDL cholesterol was higher in Indians than Caucasians.

Significance to Biomedical Research and the Program of the Institute: The findings help explain the low prevalence of coronary artery disease among southwestern American Indians, and reinforce the current notion that low levels of LDL and high levels of HDL protect against ASCVD. They also elucidate the mechanisms responsible for the low levels of LDL cholesterol observed among Southwestern American Indians.

Proposed Course: Since VLDL is the precursor of LDL and the main lipoprotein synthetic product for the liver, studies of VLDL metabolism in this population are planned. Simultaneous measurements of VLDL and LDL metabolism will be performed to further study LDL production.

Publications:

Garnick, M.D., Bennett, P.H., and Langer, T.: Low density lipoprotein metabolism and lipoprotein cholesterol composition in American Indians. J. Lipid Research 20:31-39, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54015-05 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Age of Onset of Lithogenic Bile in Southwest American Indians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. H. Bennett, Acting Chief	PCRS, NIAMDD
Other:	D. Mott, Research Biochemist	PCRS, NIAMDD
	W. C. Knowler, Staff Physician	SFSS, NIAMDD

COOPERATING UNITS (if any)
L. J. Bennion, Sacramento, California (formerly Staff Physician, PCRS, NIAMDD)
P. H. Klein, Argonne National Laboratory

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.3	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lipid composition of gallbladder bile, bile acid pool size and sex hormone levels were measured in 66 Pima Indians aged 9 to 21 years in order to determine the age of onset of lithogenic bile in this population noted for a high prevalence of cholesterol gallstones. Bile acid pool size is measured by isotope dilution using deuterated chenodeoxycholic acid, and gallbladder bile is obtained by duodenal siphonage after stimulation of gallbladder contraction. Highly saturated bile was not prevalent among Pimas under the age of 13, but bile saturation increased significantly in both sexes during pubertal growth and development. Bile saturation was 15 per cent higher in femalesthan males. Bile acid pools increased with age in the young men, but not in women. Bile cholesterol saturation correlated with obesity and urinary estrogen excretion. Highly saturated bile maybe present for several years before the onset of cholesterol cholelithiasis.

PHS-6040
(Rev. 10-76)

Project Descriptions: Z01 AM 54015-05 DD

Objectives:

1. The lipid composition of gallbladder bile, bile acid pool size and sex hormone levels were determined in Pima Indians of each sex between 9 and 21 years of age.

Methods Employed:

1. Bile is obtained by duodenal siphonage after stimulation of gallbladder contraction by intraduodenal infusion of amino acids. Chenodeoxycholic acid pool size is measured by the mass ratio of deuterated chenodeoxycholic acid to native chenodeoxycholic acid in the bile twelve hours after the intragastric administration of dideuterochenodeoxycholic acid. Determination of bile acid composition by gas-liquid chromatography permits calculation of total bile acid pool size from the chenodeoxycholic acid pool size determined as above by mass-spectrometry. Bile lipid composition is determined by standard chemical methods and expressed as percent saturation with cholesterol. Correlations of bile composition and bile acid pool size, blood levels of sex hormones, age, pubertal development, body size, sex and plasma lipoproteins are evaluated by multiple regression analysis.

Major Findings: Lithogenic bile is an acquired, rather than congenital, condition among Pima Indians. Lithogenicity of bile increases during pubertal growth and development. Bile saturation is positively correlated with obesity and urinary estrogen excretion. Bile saturation is higher in women than in men. Bile acid pool size increases during pubertal growth and development in males, but not in females.

Significance to Biomedical Research and the Program of the Institute: These results suggest that the exceedingly high rate of gallstone formation among Pima Indians is not due to an inborn defect in lipid metabolism resulting in highly saturated bile present from birth, but rather, that highly saturated bile is a trait acquired during the teen-age years. Its development correlates with increasing age and urinary estrogen excretion. The finding of more highly saturated bile in females than males suggests that the cause, and therefore the potential prevention, of the excess prevalence of gallstones among women, is in the chemical composition of their bile, rather than in gallbladder motility or some other factor related to gallstone formation. Since lithogenic bile does not develop prior to puberty, efforts to intervene in the natural history of cholesterol gallstone formation, by preventing the production of lithogenic bile, should begin with Pimas in their teen-age years. The sex difference observed in the effects of age on bile acid pool size suggests that bile acid supplementation, as with chenodeoxycholic acid, may be helpful in reducing the excess rate of gallstone formation characteristic of women. Comparison of these data to previously obtained cholecystographic data in the same Pima population suggests that there is a lag period of approximately 8 years in Pima women between the onset of lithogenic bile and the appearance of clinically detectable gallstones.

Proposed Course: The investigations are completed, the results have been published, and this project is terminated.

Publications:

1. Bennion, L.J., Knowler, W.C., Mott, D.M., Spagnola, A.M., Bennett, P.H.: Development of Lithogenic Bile During Puberty in Pima Indians. New England Journal of Medicine 300:873-876, 1979.

2. Bennion, L.J., and Grundy, S.: Risk Factors for the Development of Cholelithiasis (Part I). New England Journal of Medicine 299:1161-1167, 1978.

3. Bennion, L.J., and Grundy, S.: Risk Factors for the Development of Cholelithiasis (Part II). New England Journal of Medicine 299:1221-1227, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54016-04 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

High Density Lipoprotein Cholesterol in Indians and Caucasians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.V. Howard	Research Biochemist	PCRS, NIAMDD
Other:	P.J. Savage	Staff Physician	SFSS, NIAMDD
	P.H. Bennett	Acting Chief	PCRS, NIAMDD

COOPERATING UNITS (if any)
R.H. Unger, M.D., Univ. of Texas, Southwestern Medical School, Dallas, TX
J. Fuller, M.D. and M. Mattock, Ph.D. - Guys Hospital, London, England
D. Steinberg, M.D., U of CA, San Diego

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.2	0.4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Recent studies have implicated the importance of high density lipoprotein cholesterol as being an independent risk factor in the genesis of atherosclerosis. Studies have shown that individuals with a low amount of high density lipoprotein cholesterol tend to be at more risk for the development of ischemic heart disease and myocardial infarction. We are studying lipoprotein composition in Indians and Caucasians. Cholesterol and triglycerides are being measured in high density lipoproteins as well as other lipoprotein subclasses isolated by ultracentrifugation. These parameters are being compared in groups of normal and diabetic Indians with and without coronary artery disease, and the influence of therapy is being assessed. Apoprotein composition of specific lipoproteins will be measured. In addition, free fatty acid levels are being studied because of their precursor relationship to lipoproteins and their reflection of insulin action. From this study we will attempt to determine if diabetes is associated with changes in serum lipoproteins, and to delineate the importance of high density lipoprotein cholesterol values in the pathogenesis of ischemic heart disease.

Project Description:

Objectives:

1. Determination of lipoprotein composition in normal and diabetic Indians. Previous studies have yielded data on total serum cholesterol and triglyceride levels in the Pima Indian population. In this study lipoprotein classes will be isolated by ultracentrifugation and cholesterol and triglyceride quantitated in the isolated lipoproteins. These parameters will be compared in normal and diabetic individuals, and in diabetics before and after therapy to determine if diabetes is associated with changes in lipoprotein composition.

2. Studies of the relationship between HDL cholesterol and ischemic heart disease. Recent studies have suggested there may be a relationship between HDL cholesterol levels and atherosclerotic heart disease. Our objective is to quantify HDL levels and study HDL composition in a series of normal and diabetic Indians with and without coronary artery disease. Study of HDL in this defined population should yield information on the role of this lipoprotein in the etiology of atherosclerosis.

Major Findings:

1. Determination of lipoprotein composition in normal and diabetic Indians.

   a. Comparison of diabetics and non-diabetics. Lipoprotein composition has been compared in a group of diabetic and nondiabetic females between 20-35 years of age. Total and VLDL triglycerides were increased in the diabetics. Total and LDL cholesterol were also increased, and there were significant decreases in HDL cholesterol.

   b. Influence of diet therapy on lipoproteins. Plasma lipoprotein cholesterol and triglyceride levels were measured in 24 obese Pima Indian diabetics before and after 1-8 months on a 500 calorie diet. After the period of weight loss total plasma cholesterol had declined greater than 20% and LDL cholesterol decreased 25%. The ratio of HDL to LDL cholesterol was significantly increased in all diabetics studied. Plasma total and VLDL triglyceride levels greater than 150 mg/dl. The increases in HDL to LDL cholesterol in the diabetics correlated significantly with the changes in 2 hr. post-load glucose levels suggesting that the change in lipoprotein cholesterol distribution was induced by improved glucose tolerance. The results indicate that weight loss in maturity onset diabetics not only improves glucose tolerance but also reverses the plasma lipid changes associated with this disorder.

c. Free fatty acid levels during glucose tolerance testing in diabetics. Changes in free fatty acid and insulin levels during OGTT and IVGTT have been determined in 12 obese Pima Indians with severe diabetes, six obese Pimas with mild diabetes, 11 obese non-diabetic Pimas, and 10 obese non-diabetic Caucasians. During OGTT, the free fatty acid declines and the severe diabetics were of the same magnitude as those of the Pima and Caucasian non-diabetics (278±55 vs. 284±32 and 262±43 μEq/L). This occurred despite an insulin increment in the diabetics of only 7±2 μU/ml as compared to 252±34 and 75±18 for the Pima and Caucasian controls. IVGTT in the severe diabetics produced no insulin response and free fatty acids did not decline. In the less severe diabetics increments in insulin averaged 15±4 μU/ml during IVGTT, while free fatty decreases were 214±53 μEq/L. This was again similar to Pima and Caucasian controls whose free fatty acid changes were 234±37 and 183±51 μEq/L with insulin responses of 216±23 and 124±56 μU/ml, respectively. Assay of plasma glycerol showed declines parallel to those of free fatty acids, indicating that the free fatty acid changes in the diabetic were attributable to inhibition of lipolysis rather than to increased removal. These findings demonstrate marked sensitivity to the antilipolytic effects of insulin in individuals considered to be resistant to its glucose-lowering action.

d. Standardization of cholesterol and triglyceride assays. We have participated in a standardization program with the CDC, Atlanta, Georgia, in order to ensure that our plasma lipid measurements will be comparable to those of the Lipid Research Clinics.

e. HDL subfractions. In collaboration with Drs. Fuller and Mattock methodology has been developed for the isolation of HDL subfractions by ultracentrifugation. HDL_{2b}, HDL_{2a} and HDL_3 are isolated by sequential centrifugation of total HDL at density 1.100 and 1.125. Nine plasma samples have been fractionated by this method in both laboratories and the data are being analyzed to ensure comparability of methodology.

f. Cross sectional study of lipoprotein composition and HDL subfractions in the Pima Indians. Sampling has been initiated of all individuals over age 15 seen at biennial exams by SFSS in order to measure lipoprotein triglycerides and cholesterol. In addition, HDL subfractions are being isolated from all those between ages 35-44 years. Cholesterol and triglycerides have been measured in these fractions and they have been stored frozen for future apoprotein studies.

2. Studies on the relationship between HDL cholesterol and ischemic heart disease. Lipoprotein composition has been determined in six Indians who have had documented myocardial infarcts, and in six controls matched for tribe, age, sex, weight, and diabetic status.

Data in this small group showed no significant differences in plasma lipoproteins in the individuals with MI.

Individuals in the Pima population with evidence of EKG abnormalities have been identified and plasma samples are being obtained at the Sacaton clinic from these individuals at the time of their biennial examination. Lipoprotein cholesterol and triglycerides are being measured and HDL subfractions isolated from these and from controls matched for age, sex, weight and diabetic status.

Studies on the heparin manganese chloride method for HDL isolation indicate that plasma samples carefully frozen have identical HDL values. The length of time that plasma can remain frozen before HDL measurement is now being determined.

Significance to Biomedical Research and the Program of the Institute:

1. This study will determine whether changes in lipoprotein composition, and especially HDL cholesterol levels, are associated with diabetes and the development of ischemic heart disease. The data obtained on HDL and other lipid classes will thus help elucidate the relationship of these lipoproteins to the development of atherosclerosis.

2. In addition, this study should yield information on lipoprotein composition in groups of normal and diabetic Pima Indians. Since this population serves as a carefully defined model for adult onset diabetes, the data should contribute to an understanding of the effects of diabetes on lipoprotein metabolism and the relationship of diabetes to atherosclerosis.

Proposed Course:

1. Determination of lipoprotein composition in normal and diabetic Indians.

   a. Comparison of nondiabetics and diabetics. Assays of lipoprotein cholesterol and triglyceride in all individuals over age 15 seen for biennial exams are being continued in order to extend the findings on lipoproteins and diabetes to other sex and age groups in the normal and diabetic population. Data will also be compared to comparable Caucasian diabetics selected and assayed by Dr. Fuller at Guys Hospital.

   b. Studies on HDL. Measurements will continue of the HDL composition in 35-44 year old diabetic and non-diabetic Pima Indians. In addition, a radioimmunoassay will be developed with Dr. Fuller to measure apo A-1 and apo A-2 levels in the HDL fractions and to compare these in HDL from non-diabetics and diabetics.

c. Studies on VLDL apoproteins. Because of the rapid catabolism and low synthetic rates observed for VLDL synthesis, it is of interest to evaluate VLDL apoprotein composition. This will be accomplished in collaboration with Dr. Steinberg.

d. Effect of diet on cholesterol levels. One of the possible explanations for the lower cholesterol levels observed in the Pima Indians is that they are caused by unique aspects of their diet. A number of patients have been recently admitted to the Clinical Research Unit placed on a standardized "American" diet for a length of time similar to those of Caucasian controls. Frozen plasma samples from these patients are available and will be used to measure total and HDL cholesterol levels as well as total triglyceride levels. The results of this study will allow us to assess the role of the Pima diet on the low cholesterol levels observed in this population.

e. Free fatty acid levels. In collaboration with the contract with the University of Texas (NO1-AM-6-2219) studies are planned on the effect of low dose insulins on free fatty acid levels. The changes observed in free fatty acids will be compared to changes observed in glucose and amino acids. These studies will be conducted in diabetics and also in obese as compared to thin non-diabetic patients in order to assess insulin sensitivity in these groups.

2. Studies of the relationship between HDL cholesterol and ischemic heart disease. Studies will continue on lipoprotein composition and HDL subfractions from individuals with EKG abnormalities and controls.

We will also continue the assay of plasma lipoproteins from Indian patients with documented myocardial infarcts and matched controls.

Publications:

Howard, B.V., Savage, P.J., Bennion, L.J., Bennett, P.H.: Lipoprotein composition in normal and diabetic Pima Indians. Atherosclerosis 30:153-62, 1978

Howard, B.V., Savage, P.J., Nagulesparan, M., Bennion, L.J., Davis, M., and Bennett, P.H.: Changes in plasma lipoproteins accompanying diet therapy in obese diabetics. Atherosclerosis (in press).

Howard, B.V., Savage, P.J., Nagulesparan, M., Bennion, L.J. Unger, R.H., and Bennett, P.H.: Evidence for marked sensitivity to the antilipolytic action of insulin in obese maturity-onset diabetics. Metabolism (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54017-03 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Effects of Weight-reduction on Glucose Tolerance and Gluco-regulatory Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Bennett	Acting Chief	PCRS, NIAMDD
Other:	P.J. Savage	Staff Physician	SFSS, NIAMDD
	D. Mott	Staff Fellow	PCRS, NIAMDD
	J. Roth	Chief	D, NIAMDD
	M. Nagulesparan	Guest Worker	PCRS, NIAMDD

COOPERATING UNITS (if any)
R.H. Unger, University of Texas Health Science Center, Dallas
A.H. Rubenstein, University of Chicago, School of Medicine, Chicago

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.4	0.4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Adults with recent onset and long-duration symptomatic, ketosis resistant diabetes mellitus and with borderline abnormalities of glucose tolerance were studied with oral and intravenous glucose tolerance tests, arginine infusions and circulating mononuclear cell insulin receptor assays. Subjects were then placed on a weight reduction diet. They were restudied every 5 weeks to determine the degree of reversibility of changes in glucose tolerance and gluco-regulatory hormone secretion.

Project Description:

Objectives: Many components of the natural history of diabetes remain to be described. A major difficulty is the absence of sequential data on individuals moving from the normal to the diabetic state. Even in populations with a high prevalence of diabetes our present inability to identify subjects who will rapidly become diabetic means that large numbers of subjects would have to be studied for many years.

The study of obese subjects with borderline glucose tolerance, recent onset and long duration of symptomatic diabetes appears likely to reveal much about the interrelationship of glucose tolerance and gluco-regulatory hormone levels in a much shorter period of time. If normal glucose tolerance can be achieved by weight reduction many comparisons can be made between such subjects and subjects who have never had abnormal glucose tolerance. Comparisons can also be made between subjects who recover glucose tolerance and those who fail to respond.

The purposes of the study are:

a. To measure changes in insulin secretion which occur during the improvement of glucose tolerance which is known to occur with weight reduction in obese diabetics. To determine if the same changes occur in response to oral and intravenous glucose and to intravenous arginine infusion.

b. To measure changes in glucagon secretion which occur during improvement in glucose tolerance to determine whether the hyperstimulation and impaired suppression of glucagon seen in diabetics is reversible.

c. To determine both acute and chronic changes in insulin receptor concentrations and affinity and to evaluate whether they are solely secondary to dietary changes or affected by the presence or absence of the diabetic state.

d. To determine changes in lipoprotein concentrations and fractions which occur during improvement of glucose tolerance.

Methods Employed: Fifteen obese diabetics with onset of symptomatic glucose intolerance within the past 4 years, fifteen obese subjects with borderline glucose tolerance and fifteen long duration diabetics who have never been treated with insulin will be admitted to the clinical research center. Fifteen similarly obese non-diabetics will be admitted as controls for the effects of weight reduction itself.

Subjects will initially be placed on a weight maintaining 300 gm carbohydrate diet and have oral and intravenous glucose tolerance tests, arginine infusions with measurement of insulin and glucagon responses and measurement of insulin receptor binding to circulating mononuclear cells. Subjects will

then be placed on a 500 calorie 50 gm carbohydrate diet to induce rapid weight loss. Every fifth week subjects will be refed with a 1500 calorie 300 gm carbohydrate diet and after 4 days on the diet the initial series of tests will be repeated. Studies will be terminated when glucose tolerance stabilizes at abnormal levels or is reduced to normal levels.

Major Findings: Initial studies were conducted on 10 recent onset diabetic and 10 non-diabetic subjects. Fasting and 2 hour plasma glucose levels of the diabetics showed highly significant improvements: 260±8 to 98±4, $p<0.001$ and 412±12 to 203±10, $p<0.001$, mg/dl respectively. Insulin secretion in response to both oral and intravenous glucose improved significantly in the diabetics ($p<0.05$) while decreasing in the controls. Although the glucose disposal rate during the intravenous glucose tolerance test improved significantly in the diabetics it remained significantly below that of the controls. The acute insulin response to IV glucose increased significantly but was 12% of that seen in the controls. In contrast, insulin responses to arginine were similar in the diabetics and controls. Glucagon suppression by intravenous glucose and stimulation by intravenous arginine improved but could not be entirely normalized. Insulin binding to circulating mononuclear cells was initially similar in the two groups and increased in both following weight loss.

Studies have been completed on 8 subjects with diabetes of 6 or more years duration. In contrast to the findings in recent-onset diabetics, only 4 of the long-duration diabetics had normalization of fasting glucose levels. In addition, although some improvement in glucose tolerance occurred in all subjects, insulin secretion did not increase significantly. Despite marked improvement of insulin secretion in response to glucose, insulin secretion in response to IV arginine was still present.

Studies have also been completed on 9 subjects with borderline or impaired glucose tolerance. In contrast to both the short and long duration overt diabetics insulin secretion in these subjects was initially high and decreased following dietary therapy despite significant improvements in the glucose tolerance of the subjects.

The findings indicate that maturity onset diabetics have a selective defect of insulin secretory response to a glucose stimulus and that, early in the course of the diabetes, this defect is substantially reversible. The insulin binding studies suggest that diminished insulin sensitivity may contribute to pancreatic decompensation in individuals unable to compensate with hypersecretion of insulin. No evidence was found of a primary defect in receptor binding in maturity onset diabetes.

The overall study to date indicates that resistance to the action of insulin may be the initial lesion as glucose tolerance begins to deteriorate and that this is followed by progressive failure of insulin secretion in response to a glucose challenge.

Significance to Biomedical Research and the Program of the Institute: These studies have a good prospect of differentiating the reversible and irreversible abnormalities that characterize diabetes mellitus. It is possible that this study may identify changes that are reversible in recent onset diabetics that become irreversible with prolonged duration of hyperglycemia. These findings can be utilized to improve our methods of treating maturity onset diabetes.

Proposed Course: Additional borderline and long-duration diabetic subjects will be studied to further clarify the sequence of changes as recovery occurs. We also hope to study some ketosis resistant subjects who have required insulin therapy to control symptoms. Further studies of the relative importance of changes in insulin secretion and insulin resistance will also be conducted under project number ZO1 AM 54018-03 DD.

Publications:

1. Savage, P.J., Bennion, L.J., Flock, E.V., and Bennett, P.H.: Beta Cell Dysfunction in Maturity-onset Diabetes: Reversible Loss of Glucose-induced Insulin Secretion with Retention of Response to Arginine. In Camerini-Davalos, R.A. (Ed.): Treatment of Early Diabetes. New York, Plenum Press, 1979, pp. 219-225.

2. Savage, P.J., Bennion, L.J., Flock, E.V., Nagulesparan, M., Mott, D., Roth, J., Unger, R.H., and Bennett, P.H.: Diet-induced Improvement of Abnormalities in Insulin and Glucagon Secretion and in Insulin Receptor Binding in Diabetes Mellitus. Journal of Clinical Endocrinology and Metabolism 48:999-1007, 1979.

NIH Contract No. NO1-AM-6-2219 Starting Date: September 30, 1976
Expiration Date: August 31, 1979

Contractor: University of Texas Health Science Center
Southwestern Medical School

Money Allocated: $360,000.00 Man Years Purchased: 13

This contract enables extension of studies carried out under the NIH Project ZO1 AM 54017-03, Effect of Weight Reduction on Glucose Tolerance and Glucose regulatory Hormones.

Work to date has included consultations and measurement of glucagon, insulin lipids and lipoprotein on samples obtained by NIH personnel and one contract physician at the Phoenix Clinical Research Center. The results of some of the laboratory determinations are reported above. Results of studies on lipids and cultured cells are reported under Project ZO1 AM 54017-04. The contract physician has been engaged in the studies of the effect of diet on diabetes of long duration which are still in progress. Preliminary results indicate major differences in the degree of recovery which can be obtained in short and long duration diabetics.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54018-03 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Characterization of Metabolic and Morphologic Abnormalities in Diabetes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Bennett	Acting Chief	PCRS, NIAMDD
Other:	P.J. Savage	Staff Physician	SFSS, NIAMDD
	P. Gorden	Senior Investigator	D, NIAMDD
	J. Roth	Chief	D, NIAMDD
	B. Howard	Chemist	PCRS, NIAMDD
	D. Mott	Chemist	PCRS, NIAMDD
	M. Nagulesparan	Guest Worker	PCRS, NIAMDD

COOPERATING UNITS (if any) R.H. Unger, Univ. of Texas, Southwestern Medical School, Dallas, M.D. Siperstein, Univ. of California Medical School, San Francisco, J.R. Williamson, Washington Univ., School of Medicine, St. Louis, S.L. Aronoff, Washington Univ., St. Louis, A.H. Rubenstein, Univ. of Chiago, Chicago

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Age, sex and weight matched groups of genetically normal, prediabetic and diabetic Pima Indians and normal Caucasians were examined by means of oral and intravenous glucose tolerance tests, arginine infusions and circulatory mononuclear cell insulin receptor assays, measurement of thickness of muscle capillary basement membrane and urinary protein profiles to determine whether abnormalities of insulin, glucagon secretion or other measurements are characteristic of the prediabetic and diabetic state.

PHS-6040
(Rev. 10-76)

Project Description: Z01 AM 54018-03 DD

This investigation is a continuation and expansion of the previous projects: 1) Evaluation of the Prediabetic and Diabetic State in the Pima Indians (Z01 AM 69,003-03 EFSB), 2) Evaluation of the Prediabetic State in the Pima Indians-Caucasian Controls (Z01 AM 69,004-03 EFSB), 3) Evaluation of Basement Membrane Thickness in Diabetic Pima Indians (Z01 AM 69,005-03 EFSB).

Objectives: Many components of the natural history of diabetes remain to be described. A prospective study of genetically suspect prediabetic subjects (defined as the non-diabetic offspring of two diabetic parents) appears to be the most direct and reliable method of describing the natural history of the disease.

Study of the prediabetic will enable us to better understand the inter-relationship of glucose levels with insulin and glucagon levels, an area which has remained controversial. These studies will also clarify the relationship of hyperglycemia to microangiopathy. Detailed studies of urinary protein abnormalities and muscle capillary basement membrane thickness should clarify the history of some of the earliest physical abnormalities found in diabetes. In addition the study was designed to clarify whether technical variations are responsible for the conflicting findings of Siperstein and Williamson regarding the time capillary basement membrane thickening is first seen.

The purposes of the study are:

1. To determine whether there are abnormalities in carbohydrate metabolism gluco-regulatory hormone secretion, urinary protein excretion or capillary basement membrane thickening in non-diabetic Pima Indians compared with non-diabetic Caucasian controls.

2. To determine if there are differences in these variables between Pima Indians with 2 parents with diabetes and those with 2 non-diabetic parents.

3. To compare the two major techniques of basement membrane measurement.

4. To determine if capillary basement membrane thickening is related to the duration of carbohydrate intolerance and to the complications of diabetes.

5. To initiate a long term prospective study of prediabetes.

Methods Employed: Four groups were selected for study - Pima Indian non-diabetic offspring of two diabetic parents, non-diabetic offspring of two non-diabetic parents, subjects with long and short duration of documented diabetes and non-diabetic Caucasians with two non-diabetic parents. The non-diabetic groups were matched for age, sex and parent desirable weight.

Following dietary preparation all subjects were admitted to the Clinical

Research Center for testing. Tests included an oral glucose tolerance test, intravenous glucose tolerance test, cortisone glucose tolerance test, an arginine infusion, a gradriceps needle muscle biopsy, fundus photographs and 24-hour urine collection for determination of specific urinary proteins by immunodiffusion.

Two sub-groups of diabetics were examined a) long-term diabetics, defined as subjects with fasting plasma glucose $\geq$ 140 mg/100ml or a two hour post-carbohydrate load glucose >300 mg/100ml and >10 years duration with and without retinopathy, and b) short-term diabetics, defined as subjects who have had a documented marked deterioration in their oral glucose tolerance over the last 5 years from a two hour post-carbohydrate load plasma glucose $\leq$ 140 mg/100ml to a two hour plasma glucose $\geq$ 300 mg/100ml.

The very high prevalence of diabetes in the Pima people, while providing a very large group of genetically prediabetic individuals, raised a problem of finding individuals who are truly normal. Genetically normal Pima offspring were selected from phenotypically normal parents 45 years of age or older. It is conceivably possible, however, that with the high prevalence of diabetes in the tribe, virtually all the Pimas could carry the diabetic genome.

Because of this possibility, a group of non-diabetic Caucasians with non-diabetic family histories were examined as an additional control group to compare with the "normal" Pimas.

Results: The pattern of insulin response to oral and intravenous glucose has been claimed to be characteristic of diabetes and prediabetes. While insulin responses to oral glucose were reduced in subjects with 2 hour glucose levels over 225 mg/dl and the acute phase insulin release was absent in all diabetics there was evidence of hyperinsulinemia in the Pima non-diabetics compared to the Caucasians. The normal and prediabetic Indians had fasting and stimulated insulin levels during all the tests 2-3 times greater than those on the Caucasians. Differences in insulin levels between the two races could not be explained by differences in glucose level, age or obesity. These studies raised the possibilities of either 1) extra pancreatic insulin resistance or 2) an abnormality of biological effectiveness of Pima insulin, or 3) abnormal hepatic insulin extraction.

As part of the investigation of extra pancreatic insulin resistance, changes in free fatty acid (FFA) concentrations during oral (OGTT) and intravenous (IVGTT) glucose tolerance tests were measured in normal Pimas and Caucasians and in Pimas with severe and mild diabetes. Results are discussed under the report for project number ZO1 AM 54016-03 DD.

To assess whether hepatic insulin extraction is diminished in the Pima, serum insulin and C-peptide concentrations were determined during oral glucose tolerance tests performed on ten Pima Indians and ten Caucasians with normal glucose tolerance and a range of fasting insulin levels. The results indicate that there is no abnormality of hepatic insulin extraction in the Pimas. In addition, this study suggests that the fractional hepatic

extraction of insulin does not change over a wide range of obesity and insulin levels.

To assess the role of insulin resistance in the development of glucose tolerance, plasma glucose levels were measured in 21 non-diabetic subjects with weights ranging from normal to grossly obese during intravenous infusions of glucose insulin and somatostatin. With somatostatin blocking secretion of endogenous insulin and exogenous insulin and glucose being infused at fixed rates the level of glucose attained during the infusion is a measure of total body insulin resistance. In non-diabetics, this measure was highly correlated with both degree of obesity (γ=0.70, $p<0.001$) and fasting insulin concentration (γ=0.80, $p<0.0001$).

The method was then applied to assess the degree of insulin resistance in 15 Pima subjects with normal fasting plasma glucose but impaired oral glucose tolerance and compare it to that of 22 obesity-matched subjects with similar fasting glucose levels and normal oral glucose tolerance. Despite similar levels of insulin during the infusion (73±2 vs 78±4 μU/ml, NS), steady state glucose levels in subjects with impaired glucose tolerance (216±14 mg/dl) were significantly higher than glucose levels attained in normal subjects (139±13 mg/dl, $p<0.001$). This study indicates that a factor in addition to obesity contributes to increased resistance to glucose disposal in subjects with glucose intolerance.

Juvenile diabetes is common in Pima Indian teen-agers and ranges from mild glucose intolerance to insulin requiring diabetes. To compare Caucasian and Pima juvenile onset diabetics, 10 subjects of each race with onset of diabetes below the age of 20 years who were treated with insulin were given oral glucose tolerance tests and IV arginine infusions with measurement of plasma glucose and c-peptide concentrations. Urine was collected over 24 hour periods for measurement of c-peptide concentration as an index of 24 hour endogenous insulin secretion. Pima subjects had significantly higher c-peptide levels fasting, in response to both glucose and arginine and in 24 hour urine collections. This study suggests that even the cases of severe juvenile onset diabetes found in the Pima are a different disease from that found in the majority of young Caucasians. It also indicates that c-peptide determinations may be useful in studies of the etiology of diabetes by making it easy to distinguish classic insulin-dependent diabetics within groups of insulin treated diabetics.

Significance to Biomedical Research and the Program of the Institute: These studied are providing information on issues which have been controversial in diabetes research for over a decade. They are making is possible to systematically approach the cause of diabetes in the Pima, a group with the highest known prevalence of maturity onset diabetes.

Proposed Course: Subjects will be recalled every two years for an additional series of tests. The second recall is now in progress. This should enable careful documentation of the natural history of diabetes. Additional studies will be conducted to evaluate insulin resistance in hyperinsulinemic Pimas and to evaluate the biological activity of Pima insulin.

Publications:

1. Siperstein, M.D., Fiengold, K.R., and Bennett, P.H.: Hyperglycaemia and Diabetic Microangiopathy. Diabetologia 15:365-367, 1978.

2. Nagulesparan, M., Savage, P.J., Unger, R.H., and Bennett, P.H.: A Simplified Method using Somatostatin for the Assessment of in vivo Insulin Resistance over a Range of Obesity. Diabetes,(in press).

3. Savage, P.J., Flock, E.V., Mako, M.E., Blix, P.M., Rubenstein, A.H., and Bennett, P.H.: C-peptide and Insulin Secretion in Pima Indians and Caucasians: Constant Fractional Hepatic Extraction over a Wide Range of Insulin Concentrations and in Obesity. Journal of Clinical Endocrinology and Metabolism 48:594-598, 1979.

4. Bennett, P.H.: Letters to the Editors: The Basement Membrane Controversy. Diabetologia 16:280-281, 1979.

NIH Contract No. NO1-AM-6-2219 Starting Date: September 30, 1976
Expiration Date: August 31, 1979

Contractor: University of Texas Health Science Center
Southwestern Medical School

Money Allocated: $961,749.00 Man-years Purchased: 13

This contract is for extension of the studies carried out under the NIH Project ZO1 AM 54018-02, Characterization of Metabolic and Morphologic Abnormalities in Diabetes.

Work to date has been comparised of consultations on studies of diabetes and lipid metabolism and the measurement of glucagon, insulin, lipids and lipoproteins on samples obtained by NIH personnel and one contract physician at the Phoenix Clinical Research Center.

Studies underway utilizing contract personnel working both in Phoenix and in Dallas including further investigations of the relation of glucagon and insulin levels, attempts to restore glucagon secretion to normal by aggressive therapy of diabetes and the use of somatostatin and its analogues to investigate effects of glucagon suppression and determine extrapancreatic insulin resistance. Studies on lipid metabolism in diabetes and studies of lipoprotein turnover and interrelationships have begun. Investigation of gut hormone response to meals in Pima and Caucasian subjects are in progress.

Proposed Course: The above studies will be continued. In addition, further morphological studies of diabetic pathology utilizing electron microscopy are planned.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54019-03 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Cellular metabolism in diabetic and nondiabetic subjects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.V. Howard	Research Biochemist	PCRS, NIAMDD
Other:	P.H. Bennett	Acting Chief	PCRS, NIAMDD
	D. Mott	Research Biochemist	PCRS, NIAMDD
	P.J. Savage	Staff Physician	SFSS, NIAMDD
	H. Hidaka	Visiting Associate	PCRS, NIAMDD

COOPERATING UNITS (if any)
Joseph Larner, M.D. University of Virginia
Hitoshi Hara, M.D., Hiroshima University School of Medicine, Japan
L. Orci, M.D., University of Geneva, Switzerland

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	0.3

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Elevated insulin levels in Pima Indian adults compared to weight matched Caucasian controls suggest that cellular changes external to the pancreas may be important in the pathogenesis of diabetes mellitus. The aim of this project is to study several aspects of insulin function in cells obtained from normal and diabetic Pima Indians, in order to determine if diabetes mellitus is expressed in genetic differences at the cellular level. Fibroblast cultures have been established from normal and diabetic Pima Indians. Parameters of cell growth are being compared in the normal and diabetic cells, and insulin binding is being studied. In addition, hexose transport functions are being quantified in the presence and absence of insulin, in order to determine if cellular abnormalities in insulin response occur in individuals with diabetes.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1. Characterization of the growth of fibroblast cultures derived from genetically defined normal and diabetic Pima Indians. Previous reports of decreased cell viability and altered growth characteristics of cultured fibroblasts from diabetics indicate that the fibroblast is useful in the study of diabetes. Fibroblast cultures have been established and stored frozen in this laboratory from six genetic non-diabetic, nine genetic diabetic Pima Indians, and six normal Caucasians. Our objective is to compare parameters of growth through the life span of these cells in order to determine if adult onset diabetes is reflected in cellular growth changes. The systematic definition of growth parameters will also yield baseline information from which to interpret metabolic data.

2. Comparative metabolic properties of normal and diabetic Pima fibroblast cultures. The availability of cell cultures established from genetically characterized diabetic and non-diabetic individuals allows comparative metabolic studies designed to assess whether there are cellular differences associated with the diabetes and insulin resistance observed in the Pima population. Studies will concentrate on the effect of insulin on aspects of carbohydrate metabolism in these cells. The effect of insulin on glucose uptake and metabolism will be defined and normal and diabetic cells will then be compared in terms of their sensitivity and magnitude of response to insulin.

Major Findings:

1. Comparative studies of the growth characteristics of Pima and Caucasian fibroblasts. Comparative growth studies have been conducted using seven Pima diabetic cell lines, six non-diabetic Pima cell lines and seven Caucasian control lines. Cell density, passage number, plating efficiency, generation time, and thymidine incorporation have been monitored throughout the life span of these cultures. The results suggest that there are significant differences in cell density, passage number, plating efficiency and generation time between the Pima and Caucasian cells. However, there appear to be no significant differences between the diabetic and non-diabetic Pima cells. The results of these studies suggest that the cell cultures from this genetically homogeneous group have altered growth properties as compared to cultures from Caucasian non-diabetics.

2. Metabolic Studies:

A. Studies on insulin stimulation of glucose incorporation. Physiologic concentrations of insulin have been shown to stimulate glucose incorporation into monolayer cultures. The magnitude of stimulation is enhanced by preincubation in less than one mg/dl of glucose for at least 12 hours. Kinetic studies using 2-deoxy-glucose as a precursor indicates that glucose starvation stimulates the V max but not the Km of the hexose transport

system. Studies on the fate of the incorporated glucose indicate that under these conditions approximately 30% has been converted to glycogen, 30% remains in the acid soluble portion of the cell, predominantly as nucleotides, and 40% goes to lactate. The kinetics of the transport system have characterized using the glucose analogues 2-deoxyglucose and 3-O-methylglucose. The transport of hexoses into the fibroblasts has been shown to involve a large diffusion component. Insulin causes a small but significant increase in V max without influencing the Km.

B. Comparative studies of the response of Pima and Caucasian cells to insulin. Pima and Caucasian cells have been compared with respect to the stimulation of glucose uptake by physiological levels of insulin. Titration of the concentration of insulin required for stimulation of glucose uptake over a 20 minute period indicated half maximum stimulation at approximately 2×10^{-3} M, and there were no differences between the non-diabetic or diabetic Pima cells or between the Pima and Caucasian cells.

C. Insulin stimulation of glycogen synthase. The system developed for hexose transport has been applied to the measurement of glycogen synthase in fibroblast monolayers in collaboration with Drs. Larner and Hidaka. Stimulation of the % I form was observed in physiologic ranges of insulin; details are described in section Z01 AM 540 -01 DD.

Significance to Biomedical Research and the Program of the Institute:

1. Growth studies. The results of the growth data so far indicate that Pima fibroblasts in culture have altered growth parameters as compared to Caucasian cultures. This implies that there are fundamental differences in cell metabolism in this genetically homogeneous group and makes available a system that can be used to explore the relationship between cell parameters and the appearance of insulin resistance and diabetes in this population. The definition of growth parameters for each cell line also yields baseline values on which to evaluate comparative metabolic differences.

2. Comparative metabolic studies. Assay of cell responses to insulin should yield information on the mechanism of insulin action at the cellular level. Comparison of normal and diabetic cells will indicate if insulin resistance and/or diabetes are reflected in defects in the cellular level.

Proposed Course:

1. Growth studies: The data on growth parameters to date indicate that Pima fibroblasts have altered growth parameters compared to Caucasian controls. Growth will be systematically studied in the Caucasian and Pima cell lines in the presence and absence of insulin in order to determine if insulin has an effect on growth parameters, and whether the Pima cells respond differently than Caucasian controls. In addition, monolayer cultures will be supplied by Dr. L. Orci for electron microscopic investigation of the cell surface, in order to determine if the differences in cell density can be attributed to cell membrane changes.

2. Metabolic Studies:

A. Studies of the kinetics of hexose transport and the influence of insulin on the transport process. Insulin stimulation of glucose uptake by cultured human diploid fibroblasts will be investigated to define more completely the mechanism of insulin action on the transport system. The response to insulin will be studied as a function of the age of the culture and the stage of cell growth. The relationship between insulin's action through its own receptor versus the mitogen receptor will be clarified by determining concentration and analog specificity. In addition, the response of glucose uptake and insulin receptor regulation will be correlated.

B. Comparison of the effect of insulin on hexose transport in Pima vs. Caucasian fibroblasts. Comparative studies on insulin's glucoregulatory action will be performed in fibroblasts derived from diabetic and nondiabetic Pimas and age-matched Caucasian controls in order to determine if the insulin resistance and/or diabetes in this population can be attributed to alterations in cellular response to insulin. The sensitivity and magnitude of response of total cell glucose incorporation to insulin will be examined using ^{14}C-glucose. The nonmetabolizable glucose analogs, 2-deoxyglucose and 3-O-methylglucose will be used to compare the effects of insulin at the level of transport. These studies will be conducted under precisely standardized growth conditions so that the influence of cell growth on metabolic parameters is minimized.

C. Correlation of insulin binding and receptor regulation with hexose transport. Studies on insulin binding to human diploid fibroblasts described in section Z01 AM 54020-02 DD have shown that insulin in physiologic concentrations binds and regulates its receptor. Insulin stimulation of glucose entry and hexose transport will be correlated with binding, and the effect of down regulation of insulin receptors on the ability of insulin to stimulate hexose uptake in fibroblast monolayers will be examined. These studies will be conducted in coordination with studies of insulin action on glycogen synthase by Drs. Larner and Hidaka in order to examine this additional glucoregulatory action of insulin.

Publications:

Howard, B.V., Mott, D.M., Fields, R.M., and Bennett, P.H.: Insulin stimulation of glucose entry in cultured fibroblasts. J. Cell. Physiol. In press.

Mott, D.M., Howard, B.V., and Bennett, P.H.: Stoichiometric binding and regulation of insulin receptors on human diploid fibroblasts using physiologic insulin levels. J. Biol. Chem. In press.

delaLlera, M., Rothblat, G., and Howard, B.V.: Cell triglyceride accumulation from very low density lipoproteins isolated from normal and hypertriglyceridemic human sera. Biochim. Biophys. Acta. In press.

Macarak, E.J., delaLlera, M., Kefalides, N.A., and Howard, B.V.: A comparison of the intracellular lipids of cultured vascular endothelial cells and fibroblasts. In Vitro, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54 020-02 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Down Regulation of Insulin Receptors In Vitro in Fibroblasts and Monocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David M. Mott	Research Biochemist	PCRS, NIAMDD
OTHER:	Barbara V. Howard	Research Biochemist	PCRS, NIAMDD
	Peter J. Savage	Staff Physician	SFSS, NIAMDD
	Peter H. Bennett	Acting Chief	PCRS, NIAMDD
	Hideki Hidaka	Visiting Associate	PCRS, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An abnormality in regulation of insulin receptors in Pima Indians is being studied in-vitro using isolated circulating mononuclear cells and cultured diploid fibroblasts. Down regulation has been characterized for the fibroblast and this system is being used to identify metabolites in Pima serum which may effect receptor regulation. Investigation of fibroblasts from Pima and Caucasian subjects suggests that the Pima cells have more receptors, reduced insulin affinity and decreased down regulation. Studies to extend these observations are in progress using isolated monocytes. The effect of down regulation on the insulin sensitivity of cellular processes is being measured using normal and down regulated fibroblasts.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Previous studies on Pima circulating mononuclear cells have indicated that their excessive diabetes is not caused by abnormal insulin binding. Insulin binding in hyperinsulinemic non-diabetic Pimas, however, is not suppressed below normal Caucasian levels, indicating an abnormality in receptor regulation. This failure of elevated insulin to decrease insulin binding could be due to a defect at the cellular level or to an inhibitor in the blood. The initial objective is to determine if the abnormaility is present in isolated Pima cells free from the nutrient and hormonal influences found in-vivo or if the altered ability to down regulate is only observed when cells are exposed to serum factors. Both the isolated mononuclear cell and the cultured fibroblast can be used to detect genetic differences in insulin binding and receptor regulation between diabetic and normal Pima and Caucasian subjects. Serum from hyperinsulinemic subjects that fail to decrease their insulin binding will be tested for their effect on in-vitro insulin receptor regulation. In addition, the effect of down regulated insulin receptors on the insulin sensitivity of cellular metabolism can be measured using the in-vitro down regulated cells.

Major Findings:

1. Characterization of Insulin Binding and Down Regulation in Dipolid Fibroblasts. Cultured diploid fibroblasts bind insulin to specific receptors which can be regulated using physiologic levels of insulin. The insulin mediated regulation of insulin receptors showed a maximum decrease in insulin binding of 40% to 65%. A stoichiometric relationship between insulin binding and down regulation was indicated by the similar values obtained for the insulin dissociation constant (0.61nM) and the insulin concentration required for half maximal down regulation (0.58nM). The order of effectiveness of insulin analogues for binding and down regulation was identified: procine insulin > guinea pig insulin > proinsulin. In addition, the fibroblast down regulates in the presence of plasma providing a system for the detection of possible Pima circulating inhibitors of down regulation,

2. Insulin Receptor Studies in Pima and Caucasian Fibroblasts. A comparison of insulin binding to diploid fibroblasts from Caucasians (n=5) and Pimas (n=5) indicates that Pima cells have more high affinity receptors per cell, m=9500±2000 S.E. versus m=26000±4000 S.E. respectively. The affinities, however, for the Caucasian receptors are higher than those on the Pima cells with mean values of K_a=1.25(nM)$^{-1}$ ±0.26 S.E. versus K_a=0.59(nM)$^{-1}$ ±0.10 S.E. respectively.

In a separate study comparing down regulation in Caucasian (n=7) and Pima (n=8) fibroblasts the maximum insulin binding per cell was the same but the down regulation was less for the Pima cells (m=44%±7 S.E. versus m=65%± S.E.). These results suggest a cellular abnormality in Pima down regulation, consistent with the initial observations on circulation monocytes. Preliminary results suggest increased down regulation for Pima diabetics compared to normal Pimas.

3. In-Vitro Down Regulation in Isolated Monocytes. Isolated circulating monocytes have been shown to down regulate their insulin receptors using physiologic levels of insulin. Although this system has not been fully characterized, monocytes from both Pimas and Caucasians have shown sensitivity to procine insulin in the down regulation assay.

Significance to Biomedical Research and the Program of the Institute.

The in-vitro demonstration of down regulation using physiologic insulin concentrations will permit investigation of the mechanisms for insulin regulation of cellular metabolism on samples from insulin resistant subjects. The studies on monocytes and fibroblasts from Pimas with abnormal down regulation should clarify the importance of insulin receptor regulation in insulin resistance and the associated diabetes and obesity of the Pima.

Proposed Course:

1. Characterization of Insulin Binding and Down Regulation in Diploid Fibroblasts. The methodology established for assaying insulin down regulation of insulin receptors is being used to detect other metabolites which may participate in this regulatory mechanism. Selected Pima and Caucasian plasmas are being assayed for possible differences in levels of inhibitors of down regulation. In order to assess the importance of down regulation in effecting the insulin sensitivity of a variety of cellular events, insulin stimulation of transport processes and intracellular metabolism is being measured on control and down regulated cells. Methodology for insulin binding to the fibroblasts in monolayer is being established for comparisons of the extent of insulin binding with the percent maximum stimulation of insulin sensitive cellular metabolism. The analysis of insulin stimulation of glycogen synthase, alpha-aminoisobutyrate uptake, pyruvate kinase, and $(Na^+ + K^+)$ATPase are described in a separate report.

2. Insulin Receptor Studies in Pima and Caucasian Fibroblasts. Insulin receptor number per cell and affinity are being measured on additional Pima and Caucasian fibroblasts in order to clarify suggested genetic differences in these parameters. Methods for solubilizing and purifying insulin receptors from diploid fibroblasts are being established. Purified insulin receptors from Pima and Caucasian cells will be characterized for possible structural differences suggested by their different affinities for insulin. Initial studies will use polyacrylamide gel electrophoresis to compare different receptor preparations. Additional fibroblasts from normal and diabetic Pimas are being characterized for possible genetic differences in their extent of down regulation of insulin receptors.

3. In-Vitro Down Regulation in Isolated Monocytes. The methodology is being established for a comparison of insulin binding to freshly isolated monocytes and to the same patient's monocytes following in-vitro down regulation. Results from this study will distinguish between a cell or serum based abnormality responsible for differences in Caucasian and Pima receptor regulation.

Publications:

Mott, D.M., Howard, B.V., and Bennett, P.H.: Stoichiometric Binding and Regulation of Insulin Receptors on Human Diploid Fibroblasts Using Physiologic Insulin Levels. Journal of Biological Chemistry In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54021-02 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Hemoglobin A1 Levels in Diabetes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Bennett	Chief	EFSB, NIAMDD
Other:	E.V. Flock	Visiting Scientist	PCRS, NIAMDD
	P.J. Savage	Staff Physician	SFSS, NIAMDD
	W.C. Knowler	Staff Physician	SFSS, NIAMDD

COOPERATING UNITS (if any)

N. Rushforth, Case Western Reserve University, Cleveland, Ohio

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Glycosylated hemoglobin concentrations were measured in 400 Pima Indians, aged 15 to 91 years, who were participating in the epidemiological studies of diabetes in the Gila River Indian Reservation in Arizona. At the same time plasma glucose levels, fasting and two-hours post-glucose load (75 g), were determined and compared with the hemoglobin to see which test might be the best indicator of the presence of diabetes. Previously the glucose levels, have been described by a model of two overlapping Gaussian distributions. Attempts were made to fit the same model to the glycosylated hemoglobin concentrations. In those aged 25 years and over, the glycosylated hemoglobins were bimodally distributed and like the glucose levels were a satisfactory fit to the overlapping Gaussian distribution model. These observations indicate the presence of two subpopulations in the Pima Indians, one normal and one diabetic. In preliminary studies, levels of glycosylated hemoglobin showed no better association with complications of diabetes than fasting and post-load glucose levels.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The present study was designed to examine the feasibility of using glycosylated hemoglobin determinations to examine the relationship between vascular complications and hyperglycemia. We have also examined the frequency distributions of glycosylated hemoglobin levels to determine if they could be used to separate the diabetic and non-diabetic subgroups within a population.

Methods Employed: Glycosylated hemoglobin (HG A1) was measured by a modification of the method of Trivelli, involving washing the red cells with physiologic saline, hemolyzing the cells with water and toluene, dialyzing the hemolyzate against a cyanide-phosphate buffer and then chromatographing the hemolyzate on a Bio-Rex 70 column. The glycosylated hemoglobin was eluted with a cyanide-phosphate buffer (pH 6.7) and then the rest of the hemoglobin was eluted with a phosphate buffer (pH 6.4). The optical density of the two fractions was measured at 415 nm and the percentage of the total hemoglobin that was glycosylated was calculated. The frequency distributions of glycosylated hemoglobin in the 15 to 24 and 25 and over age groups were examined and compared with the fasting and two-hour glucose concentrations. Maximum likelihood methods were used to determine the mean and standard deviations of the hemoglobin distributions.

Major Findings: A single distribution of glycosylated hemoglobins was found in the young males, aged 15 to 24 years. In the young females single mode was seen corresponding to the normal population with a few outlying values indicating the presence of a small number of diabetics in this age group. In those aged 25 and over in both males and females the hemoglobin levels were bimodally distributed with a satisfactory fit to the overlapping Gaussian distribution model previously applied to the frequency distribution of plasma glucose levels in the Pima population. The normal components of the distribution had mean levels ranging from 10.1 to 10.6% with a slight increase in mean level with increasing age. The diabetic component had mean levels averaging 17.5%. The cut-off points ranged from 13.5 to 14%. Distinct elevation of glycosylated hemoglobin levels did not occur until fasting hyperglycemia ($\geq$140 mg/dl) was present. Glycosylated hemoglobin levels are highly correlated with both fasting and post load plasma glucose levels and will not discriminate better than glucose measurements between non-diabetic and diabetic subjects. Triglyceride concentrations showed only low order correlations with glycosylated hemoglobin levels. Retinopathy was confined almost entirely to subjects with elevated glycosylated hemoglobin levels.

Significance to Biomedical Research and the Program of the Institute: The presence of two subpopulations in the Pima Indians, one normal and one diabetic is clearly indicated by the frequency distributions of glycosylated hemoglobins, fasting glucose levels and two-hour glucose levels. The determination of glycosylated hemoglobin can be useful for epidemiological studies where the performance of glucose tolerance testing is impractical.

Proposed Course: This project will be continued particularly in the 35 and older age group to further elucidate changes in the bimodal distribution of hemoglobin A1 with aging. In addition, further studies of the association between hemoglobin A1 levels and the prevalence and incidence of diabetic complications will be conducted.

Publications:

Flock, E.V., Bennett, P.H., Savage, P.J., Webner, C.J., Howard, B.V., Rushforth, N.B., and Miller, M.: Bimodality of Glycosylated Hemoglobin Distribution in Pima Indians: Relationship to Fasting Hyperglycemia. Diabetes (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54022-02 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mechanism of the Lithogenic Effects of Oral Contraceptives

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Bennett, Acting Chief	PCRS, NIAMDD
Other:	D.M. Mott, Research Biochemist	PCRS, NIAMDD
	B.V. Howard, Research Biochemist	PCRS, NIAMDD
	W.C. Knowler, Staff Physician	SFSS, NIAMDD

COOPERATING UNITS (if any)

L.J. Bennion, Sacramento, California (formerly Staff Physician, PCRS, NIAMDD)

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To determine whether the effects of oral contraceptives in increasing the cholesterol saturation of bile are mediated by increased secretion of cholesterol or by decreased secretion of bile acids and phospholipids, we measured the rate of biliary secretion of these lipids in 5 normal women during oral contraceptive usage and again during normal menstrual cycles on no medication. We also measured bile acid pool size and bile acid composition, we well as lipid composition and percent cholesterol saturation of gallbladder bile. These findings indicate that oral contraceptive usage increases biliary cholesterol secretion without changing bile acid pool size or bile acid composition, thereby raising the level of cholesterol saturation of bile and predisposing to cholesterol precipitation and gallstone formation.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To elucidate the mechanisms by which the ingestion of oral contraceptives results in the production of highly saturated bile and cholesterol gallstones.

Methods Employed: Gallbladder bile is obtained by duodenal siphonage after stimulation of gallbladder contraction. Bile acid pool size is determined by isotope dilution using radiolabelled cholic acid. Secretion of cholesterol, bile acid and phospholipid from the liver into the bile is measured by marker dilution using a three lumen tube: beta-sitosterol is infused through the most proximal tube into the duodenum at a constant rate; bile is sampled 10-12 cm distal, after complete mixing of the marker with cholesterol emerging from the ampulla of Vater. Hepatic bile composition is determined from bile aspirated at the ampulla of Vater through a third lumen.

Major Findings: Oral contraceptives usage results in a disproportionate increase in cholesterol secretion, with no consistent pattern of alteration in bile acid pool size or secretion rate.

Significance to Biomedical Research and the Program of the Institute: These findings suggest that estrogens, and/or progestins, produce cholesterol hypersecretion when administered as oral contraceptive pills. This cholesterol hypersecretion results in saturated bile and in an increased rate of cholesterol gallstone formation among users of oral contraceptives. Administration of chenodeoxycholic acid, which reduces biliary cholesterol secretion, might be expected to prevent this side-effect.

The estrogens and progestins secreted by the ovaries of normal women between puberty and menopause may act through a similar mechanism to produce the excess frequency of cholesterol gallstone disease observed among women.

Proposed Course: These studies have been completed and the project is inactive.

Publications:

1. Bennion, L.J., Mott, D.M., Howard, B.V.: Mechanism of the Lithogenic Effect of Oral Contraceptives: Enhanced Biliary Cholesterol Secretion. Metabolism (in press)

2. Bennion, L.J., Mott, D.M., Howard, B.V.: Oral Contraceptives Raise the Cholesterol Saturation of Bile by Increasing Biliary Cholesterol Secretion. Metabolism (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 54 023-02 DD

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Very low density lipoprotein metabolism in Pima Indians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.V. Howard	Research Biochemist	PCRS, NIAMDD
Other:	P.J. Savage	Staff Physician	PCRS, NIAMDD
	D. Mott	Research Biochemist	PCRS, NIAMDD
	P.H. Bennett	Acting Chief	PCRS, NIAMDD
	L. Zech	Staff Physician	IR MD, NHLBI

COOPERATING UNITS (if any)
David Bilheimer, M.D., Dept. of Internal Medicine, Univ. of Texas, Dallas, TX
Scott M. Grundy, M.D., Dept. of Medicine, School of Medicine, Univ. of CA, San Diego, CA

LAB/BRANCH
Digestive Diseases Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Pima Indians have been shown to have a high prevalence of diabetes mellitus and hyperinsulinemia. Despite these the Pimas have greatly decreased incidence of cardiovascular disease. Studies of plasma lipids have shown that total serum cholesterol values are low, and they do not increase with age. Plasma LDL levels are low and LDL synthetic rates were shown to be decreased. VLDL metabolism in Pima Indians will be investigated in order to study the relationship between VLDL and the other serum lipoprotein classes and to determine the effect of hyperinsulinism on VLDL metabolism. Synthetic and catabolic rates for VLDL will be compared in groups having different insulin levels using tritiated glycerol as a precursor. VLDL metabolism will be studied in a group of diabetics to determine the influence of hyperglycemia on VLDL metabolism in this population. Conversion of VLDL to LDL will be studied using ^{125}I VLDL and ^{131}I LDL simultaneously. The results of this study should yield data on VLDL metabolism and its conversion to LDL in this population and explore the relation between these lipoproteins and cardiovascular disease.

Project Description:

Objectives:

1. The metabolism of VLDL will be studied using a pulse injection of H glycerol. This will yield values for synthetic and catabolic rates of VLDL which can be compared to those of Caucasians assayed under similar conditions.

2. The relation between plasma insulin levels and VLDL synthetic and catabolic rates will be determined by ^{3}H glycerol VLDL studies in Pimas having differing serum insulin levels.

3. VLDL metabolism will be studied in a group of untreated male diabetics matched in age and weight to group #1. Effect of hyperglycemia on synthetic and catabolic parameters will be assessed.

4. The conversion of VLDL to LDL will be studied in a group of Pima Indians using I^{125} and I^{131} LDL. This study will yield an independent measure of VLDL metabolic rates, and also show the extent of conversion of VLDL to LDL in this population.

5. Plasma free fatty acids, which are the immediate precursors of the fatty acids in VLDL triglyceride, will be quantified and turnover will be assessed by a pulse injection of albumin bound ^{14}C free fatty acid. Free fatty acid levels and turnover rates will be compared in groups having low and high insulin levels to determine if adipose tissue free fatty acid production reflects or correlates with fasting serum insulin values.

6. Post heparin lipolytic activity will be determined in a small number of Pima Indians to determine if the VLDL metabolism in the Pimas is related to the levels of this enzyme.

Major Findings:

^{3}H Glycerol Studies. Fifteen nondiabetic male Pima Indians (averaging 170% ideal weight) have been admitted for VLDL studies using ^{3}H glycerol, and the specific activity curves have been analyzed by Dr. Loren Zech to yield kinetic data on synthesis and catabolism. Results have been compared to a group of 10 obese normolipidemic Caucasians (163% ideal weight) and 13 normal weight Caucasians (100% desirable weight) studied under identical conditions by Dr. Scott Grundy. Whereas obese Caucasians had greatly elevated VLDL-Tg synthetic rates, VLDL-Tg synthesis in the obese Pimas was significant lower, and similar to that of normal weight Caucasians. VLDL-Tg pools in the Pimas were lower than either Caucasian group, and the FCR was significantly higher.

When the relationships between plasma insulin levels were examined, insulin levels were not correlated with either VLDL-Tg pools, FCR, or synthesis. Further analyses of the data indicated VLDL-Tg synthesis in this population may be inversely related to free fatty acid declines.

The IDL fraction (density 1.006-1.019) has been isolated from 8 of these patients, and the specific activity data from this fraction is being used for the development of a multicompartmental model of IDL metabolism.

^{125}I studies and free fatty acid metabolism. No patients have been admitted for these objectives.

Significance to Biomedical Research and the Program of the Institute: The results of these studies are relevant both to in vivo lipoprotein conversions, and the relationship of diabetes to these processes. The studies on the interconversions of VLDL to LDL will determine why LDL synthesis is low in the Pimas. The comparison of VLDL metabolism in populations with high and low insulin levels will indicate whether or not increases in serum insulin lead to increased synthesis of VLDL or VLDL triglyceride. Furthermore, a comparison of VLDL and LDL turnover in Caucasians with that of the Pimas will yield information on the relationship between serum lipoprotein turnover and cardiovascular diseases.

Proposed Course:

^{3}H Glycerol studies: VLDL-Tg metabolism will be studied in a group of 10 male diabetic Pima Indians matched in age and weight to the previous group of nondiabetics. Diabetics will be untreated with 2 hour plasma glucose levels greater than 250 mg/dl. Data will be analyzed to determine the influence of hyperglycemia on VLDL-Tg metabolism in this population.

Isolation of IDL fractions will continue and specific activity data used for further development of IDL model and analyses of IDL metabolism on this population.

Studies of objectives 4-6: These will await the completion and analyses of the ^{3}H glycerol studies.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 54 024-01 DD

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

In-Vitro Insulin Resistance in Pima Indians

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David M. Mott	Research Biochemist	PCRS, NIAMDD
Other:	Barbara V. Howard	Research Biochemist	PCRS, NIAMDD
	Hideki Hidaka	Visiting Associate	PCRS, NIAMDD
	Peter H. Bennett	Acting Chief	PCRS, NIAMDD

COOPERATING UNITS (if any)

Joseph Larner, University of Virginia Medical School, Charlottesville, VA
Hitoshi Hara, Hiroshima University School of Medicine, Japan

LAB/BRANCH

Digestive Diseases Branch

SECTION

Phoenix Clinical Research Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Physiologic insulin levels stimulate glycogen synthase in diploid fibroblasts. The percent maximal stimulation of this activity is being compared to fractional receptor occupancy in fibroblasts from normal and diabetic Pima and Caucasian subjects. This in-vitro measure of the insulin resistance of a specific activity will also be applied to insulin stimulation of (Na^++K^+)ATPase, pyruvate kinase and alpha-aminoisobutyrate transport. Fat cells from obese and normal subjects are being sized and compared in terms of the antilipolytic effects of insulin.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives.

1. Relationship Between Insulin Binding and Stimulation of Metabolic Activity. Our primary objective is to compare the extent of insulin receptor occupancy with the stimulation of a variety of insulin sensitive metabolic functions in genetically characterized diploid fibroblasts from diabetic and normal Pima and Caucasian subjects. Measurements of insulin stimulation will be made on glycogen synthase, pyruvate kinase, $(Na^+ + K^+)$ATPase and alpha-aminoisobutyrate transport.

2. Mechanisms of Metabolic Stimulation by Bound Insulin. A comparison will be made between the kinetics of insulin binding and insulin stimulation of a variety of metabolic activities in a single cell line. Fractional receptor occupancy required for half maximal insulin stimulation of the different activities will be determined.

3. Lipid metabolism will be compared in fat cells from thin and obese patients to determine if hyperinsulinemia in Pima Indians could alter lipolysis and contribute to their obesity.

Methods:

1. Glycogen synthase is measured as ^{14}C-glucose uptake into ethanol precipitable glycogen.

2. Pyruvate kinase is measured using lactic dehydrogenase in a coupled enzyme assay producing NAD from NADH.

3. α-aminoisobutyrate uptake is measured using the fritium labeled amino acid analog.

4. $(Na^+ + K^+)$ATPase activity is measured as the ouabain sensitive release of inorganic phosphate.

5. Insulin binding is measured on monolayer or suspended fibroblasts using ^{125}I-insulin.

6. Methods have been established for fat cell sizing and insulin inhibition of fat cell lipolysis. Techniques developed on cells from rat epididymal fat pads have been applied to human biopsies obtained at the time of cholecystectomy.

Major Findings:

1. Glycogen Synthase: Glycogen synthase activity in diploid fibroblasts is stimulated about 100% above basal following a 15 minute incubation with insulin. Stimulation is observed at physiologic insulin concentrations

(10^{-10}M) and 2 to 3x10^{-9}M insulin is required for half maximal increase in glycogen synthase activity.

2. Human Adipocytes: Preliminary results indicate that compared to rat adipocytes, human cells have an increased size range accompanied by reduced isoproterenol stimulation of lipolysis.

Significance to Biomedical Research and the Program of the Institute.

1. Relationship Between Insulin Binding and Stimulation of Metabolic Activity. Preliminary studies of insulin binding to diploid fibroblasts from Pima and Caucasian subjects indicate that the maximum specific binding at low insulin concentration is similar but at levels above 0.2M Pima cells bind more insulin. This difference in binding is a result of an increased receptor number which more than compensates for the decreased affinity at this insulin concentration. (See Project Number Z01 AM 54 020-02 DD). Increased insulin binding in-vitro to cells from an insulin resistant Pima subject raises a question concerning the relationship between the extent of insulin binding and the resulting level of insulin stimulated metabolic functions. Comparisons of receptor occupancy and metabolic responses to insulin should yield information on the mechanism of insulin action at the cellular level. Comparison of normal and diabetic cells will indicate if insulin resistance and/or diabetes are reflected in defects at the cellular level.

2. Mechanisms of Metabolic Stimulation by Bound Insulin. The multiplicity of mechanisms by which bound insulin enhances a variety of cellular activities is not known. A comparison of the kinetics for insulin binding and activation of a variety of metabolic activities in one type of cell should elucidate this poorly defined system.

Proposed Course:

1. Insulin binding and activation of glycogen synthase will be studied over a range of insulin concentrations in order to determine the percent receptor occupancy required for various levels of enzyme activity. These studies will be performed on representative normal and diabetic Pima and Caucasian fibroblast lines.

2. Assays for basal and insulin stimulated activity of pyruvate kinase, (Na^++K^+)ATPase and alpha-aminoisobutyrate will be developed for subsequent comparison with fibroblast insulin receptor occupancy.

3. Insulin resistance measured in terms of receptor occupancy per level of insulin stimulated activity will be compared in diabetic and normal Pima and Caucasian lines measuring (Na^++K^+)ATPase, alpha-aminoisobutyrate transport and pyruvate kinase.

4. Human adipocytes from obese and normal patients will be characterized in terms of size and sensitivity to the antilipolytic effects of insulin.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER 201 AM 54 025-01 DD

PERIOD COVERED
October 1, 1978 to September 30. 1979

TITLE OF PROJECT (80 characters or less)
(Na^++K^+)ATPase Activity in Obese Subjects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David M. Mott	Research Biochemist	PCRS, NIAMDD
Other:	Barbara V. Howard	Research Biochemist	PCRS, NIAMDD
	Peter J. Savage	Staff Physician	SFSS, NIAMDD
	Peter H. Bennett	Acting Chief	PCRS, NIAMDD

COOPERATING UNITS (if any)
None.

LAB/BRANCH
Digestive Disease Branch

SECTION
Phoenix Clinical Research Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85016

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Erythrocyte (Na^++K^+)ATPase activity has been measured in Caucasian and Pima Indian subjects. Obese Pima subjects have a significantly reduced level of enzyme activity compared to normal or obese Caucasians. Additional studies will determine the importance of this lesion as a genetic factor which promotes obesity. For this purpose, the activity will also be measured in diploid fibroblasts from thin and obese Pima Indian and Caucasian subjects.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Pima Indians are a genetically homogeneous group characterized by a high incidence of obesity and hyperinsulinemia. Although a genetic basis for this obesity has not been established, the ob/ob mouse may serve as an animal model for Pima obesity. The ob/ob mouse has been found deficient in basal and thyroid inducible (Na^++K^+)ATPase. The importance of this enzyme activity has been established for the thyroid stimulation of oxygen consumption. A deficiency of (Na^++K^+)ATPase activity could make more calories available for stored fat since less energy is expended on ATPase activity. A deficiency in the induction of a single enzyme therefore, may explain the obesity of the ob/ob mouse. If this explanation for obesity applies to humans, obese subjects with normal thyroid function should have decreased plasma membrane (Na^++K^+)ATPase activity and a diminished response of this enzyme to triiodothyronine. In-vitro basal and triiodothyronine stimulated (Na^++K^+)ATPase activities will be compared in erythrocytes from thin and obese subjects with normal thyroid function. Results of this study will be used to assess the extent that an abnormal regulation of energy balance is responsible for obesity in Pima and weight matched Caucasian subjects.

Methods: (Na^++K^+)ATPase activity is determined in erythocytes as the ouabain sensitive release of inorganic phosphate.

Major Findings: (Na^++K^+)ATPase activity per grams of hemoglobin has been measured in red cells from normal and obese Caucasians and obese Pimas. Preliminary results indicate that no differences in activity exist between normal and obese Caucasians. Obese Pimas (n=5) however, have a mean activity which is significantly ($p<0.001$) lower than the mean Caucasian (n=8) (Na^++K^+)ATPase level (0.52$\pm$0.08 v.s. 0.88$\pm$0.09, uM phosphorus per gram Hb per hour, 37°C$\pm$S.E.M.)

Significance to Biomedical Research and the Program of the Institute.

The demonstration of abnormally low red cell (Na^++K^+)ATPase activity in obese Pimas compared to obese and normal Caucasians suggests that a genetic lesion effecting enzymatic catalysis of ATP hydrolysis could contribute to the excessive obesity in the Pima population. Studies on additional red cell samples as well as other tissues will indicate the prevalence of this abnormality in the Pima population and its casual relationship with obesity.

Proposed Course:

1. Red cell (Na^++K^+)ATPase activity will be determined on 60 euthyroid subjects divided equally among obese and thin Pimas and Caucasians. These results should clarify the association of low (Na^++K^+)ATPase activity with race and obesity.

2. If Pima obesity is associated with low (Na^++K^+)ATPase activity, the red cell assay will be used to study the natural history of this lesion using pre-obese Pima subjects.

3. The (Na^++K^+)ATPase activity will be measured in diploid fibroblasts from thin and obese Pima and Caucasian subjects. A comparison of this activity in a variety of cells cultured in a uniform environment should clarify possible genetic associations with obesity.

Publications:

None.

ANNUAL REPORT - 1978-1979

CLINICAL ENDOCRINOLOGY BRANCH

Reorganization of the Branch into three Sections was accomplished during this year. The Section on Endocrine Biochemistry is now under the direction of Dr. Jan Wolff. The new Section of Endocrine Physiology is headed by Dr. Jacob Robbins, and the new Section on Protein Structure is directed by Dr. Harold Edelhoch. Exchanges between CEB and laboratories in France and Italy continued; Dr. Gavaret of Hopital Bicetre, Paris, and Dr. Formisano of the University of Naples spent four to nine months in Bethesda; and Dr. Edelhoch made an extended visit to their departments in Europe. Other visiting scientists in CEB during the year were from laboratories in Finland, Japan and Switzerland.

I. Thyroid Biochemistry

A. Thyroid Cell Membrane and Mechanism of TSH Action

Although previous studies showed that adenosine has both stimulatory and inhibitory effects on adenylate cyclase in the adrenal and in several other cells, in thyroid membranes only the inhibitory effect has been demonstrable. This was observed in three species, including man, and was effective when cyclase was activated by TSH or several other activators. Manganous ion, however, which stimulates adenylate cyclase, enhanced the inhibition by adenosine. Purine-modified analogs of adenosine were inactive or showed slight stimulation. Thus, thyroid membranes contain primarily the "P" (purine) site and little "R" (ribose) site activity. The "P" site appears to be associated with the catalytic unit since it persists in solubilized cyclase (Wolff, Cook).

Further study of the solubilized adenylate cyclase from beef thyroid membranes showed that the fluoride-activated enzyme has an apparent molecular weight of 120,000 daltons. Since this is smaller than the 160,000 molecular weight previously observed when the activator was Gpp(NH)p, it appears that the GTP-binding protein is lost from the fluoride-activated preparation (Wolff, Goldhammer).

In studies of TSH binding to bovine and human thyroid cells, purified thyroid cell membranes and human lymphocytes, the existence of two different binding sites has been demonstrated. The number of high affinity, high specificity sites is increased by removal of sialic acid, but removal of galactose, N-acetyl glucosamine and fucose had little additional effect. More complete deglycosylation, however, decreased TSH binding (Weintraub, Pekonen).

B. Thyroid Hormone Secretion

For the study of the role of microtubules in thyroid hormone secretion, iodinated tubulin was prepared in order to measure its interaction

with cell organelles. Iodination, however, inhibited copolymerization with native tubulin. In the course of this work, it was discovered that self-iodinated lactoperoxidase (LPP) polymerized with tubulin, and bound to the 30-36S, ring-shaped oligomers of tubulin. One mole of LPO interacted with a tubulin dimer. This unexpected interaction can be exploited for the affinity chromatography of microtubule subunits and for the labeling of cell organelles in ultramicroscopy (Rousset, Wolff).

B. Thyroid Hormone Synthesis

When thyroxine is formed by the intramolecular coupling of two diiodotyrosyl residues in thyroglobulin, one side chain is separated from the tyrosine which forms the phenolic ring and remains within the peptide chain as a new amino acid. The nature of this "lost side chain" has never been clarified. By studies with ^{14}C-tyrosine-labeled thyroglobulin, it was shown that the hydrolysate of thyroglobulin iodinated in vitro contained ^{14}C-pyruvic acid which increased in proportion to the thyroxine content. This pyruvic acid most likely was derived from dehydroalanine during hydrolysis (Cahnmann, Gavaret).

Chemical methods for the preparation of high specific activity labeled thyroid hormones were also devised to obtain labeled substrates for metabolic studies. By iodination of [3,5-^{125}I]-diiodothyronine under appropriately designed conditions, the product was mainly either thyroxine or triiodothyronine. At pH 11, a large yield of T_3 labeled in the nonphenolic ring was obtained (Cahnmann, Sato).

C. Thyroxine-Protein Interactions

Thyroxine-binding globulin (TBG), the major thyroxine transport protein of serum, is a glycoprotein containing 23% carbohydrate. To study the role of the oligosaccharide chains on the structure and function of TBG, 87% of the sugar was removed by treatment with mixed glycosidases. This had no effect on the stoichiometry of T_4-binding and caused only a three-fold decrease in affinity for T_4. Only a two-fold decrease in affinity of TBG for anti-TBG antibody was observed. Thus, the carbohydrate moiety is not involved in hormone-binding or in the antigenic determinants on the protein (Cheng, Robbins).

The thyroxine-binding sites of TBG, prealbumin (PA) and albumin were investigated by the method of electron spin resonance (ESR) employing oxytetramethylpyrrolinyl derivatives of thyroxine. The depth of the TBG and PA sites were similar (20Å), but the albumin site was deeper. Furthermore, the ligand was held less rigidly in the TBG site compared to those of PA and albumin even though the affinity of the TBG site is very much greater (Cheng, Robbins).

D. Thyroid Hormone-Cell Interactions

Rhodamine-labeled thyroid hormones were prepared and used to study their interaction with cells observed under a fluorescence microscope

utilizing video intensification. (This is a collaborative project with Dr. Ira Pastan, NCI.) The retention of biological activity by these derivatives was proved by demonstrating that they bind to the specific hormone receptor sites in liver cell nuclei. When monolayer cultures of pituitary tumor cells (GH_1) were exposed to Rho-T_3, the hormone was bound to the cell surface in patches, later forming a cap, then entering the cytoplasm in vesicles, and still later appearing in the nucleus. These phenomena are under study to evaluate their possible relevance to the mechanism by which thyroid hormones enter cells and reach their intracellular sites of action (Cheng, Pastan, Robbins).

The fluorescence properties of rhodamine-iodothyronine derivatives were studied in order to understand the mechanism by which the rhodamine fluorescence is quenched in the hormone derivative and then is restored when the complex is bound to protein or cell membranes. The results indicate that rhodamine-T_3 exists in water at low temperature in a folded form which facilitates transfer of the rhodamine fluorescence to the iodothyronine moiety. Binding of the iodothyronine perturbs this intramolecular folding and results in fluorescence of the rhodamine chromophore (Edelhoch, Chen, Irace).

E. Thyroid Hormone Metabolism

The monodeiodination of thyroxine and related iodothyronines in cultured monkey hepatocarcinoma cells differs from that of normal liver since nonphenolic ring deioidination is much more active than phenolic ring deiodination whereas studies of this enzyme have been hampered by its low activity in normal liver. It was found that the extent of this reaction is markedly increased when cell division is inhibited by culturing the cells in an amino-acid deficient medium. Studies in cell homogenates proved that this effect resulted from an abrupt increase in enzyme synthesis. No change in enzyme Km occurred, nor was there evidence for cofactor changes. The quantitative difference in response of the two deiodinases (the effect on nonphenolic ring deiodination was much greater) provides further evidence that they are separate enzymes (Robbins, Sato).

The localization of the nonphenolic ring deiodinase within the hepatocarcinoma cell has been investigated. It has been reported that this enzyme in normal liver is in the cytosol whereas the phenol ring deiodinase is located in the plasma membrane. Our studies show that the nonphenolic ring deiodinase is in the membrane-bound fraction of the carcinoma cells. More precise localization has not yet been achieved since the plasma membrane marker enzymes are markedly deficient in these cultured cells (Robbins, Goldman).

F. Thyroid Hormone Actions

The administration of thyroid hormones causes an increased level of many hepatic enzyme activities, suggesting that a general increase of

many liver proteins may occur. This was investigated in the subcellular fractions of rat liver analyzed by 2-dimensional gel electrophoresis. In mitochondrial extracts, 150 protein spots could be recognized reproducibly. A limited number of these were rapidly increased in amount when T_3 was given to hypothyroid animals. One protein was seen only after T_3. Another group of proteins decreased in amount (Rall, Villki, Gavaret).

G. Studies of Thyroid Disease

In the management of patients with thyroid cancer, it is customary to allow hypothyroidism to develop in order to test for the presence of functioning metastases. In a study of more than 30 patients, it was found that two weeks after stopping T_3, before serious hypothyroid symptoms develop, detection of thyroid remnants and metastases by ^{131}I scanning are as good as after four weeks. In comparison with our previous studies, it was shown that addition of thyrotropin-releasing hormone (TRH) to the testing regimen is unnecessary (Robbins, Goldman).

Serum TSH and its subunits were measured in the serum of T_3-suppressed patients during the development of hypothyroidism following withdrawal of T_3. The initial event was found to be the appearance of high levels of the alpha subunit. As hypothyroidism progressed, the TSH-beta subunit and TSH increased and alpha subunit decreased. These findings resemble the development of pituitary function in the fetus, and indicate that the synthesis of beta-subunit is rate-limiting in TSH biosynthesis (Weintraub, Goldman).

In two additional patients with the syndrome of inappropriate TSH secretion, the metabolism of ^{125}I-labeled T_4 was studied. In one patient, in whom there was peripheral as well as pituitary resistance to thyroid hormone, the metabolic clearance of T_4 was prolonged and the conversion of T_4 to rT_3 was markedly decreased. This phenomenon may be useful as a sensitive indicator of peripheral resistance to thyroid hormone (Weintraub, Fein).

Further improvement in the assay of TSH receptor antibodies in patients with thyroid disease has been achieved. Treatment of thyroid membranes with proteolytic inhibitors and high salt concentrations increases the sensitivity of the assay while retaining its specificity. Immunoglobulin preparations from patients with Graves' disease showed TSH-binding inhibition in 80% of cases. In contrast, only one of fourteen patients with hyperthyroidism due to inappropriate TSH secretion had TSH-binding inhibition (Weintraub, Pekonen).

Five patients with a new syndrome of autoimmune thyroiditis and transient hypothyroidism have been identified. This syndrome occurs in the postpartum period with a characteristic course. Transient hyperthyroidism occurs two to three months postpartum, followed within another two to months by hypothyroidism and goieter, both of which resolve within one year. There are no symptoms of subacute thyroiditis, and fine-needle aspiration biopsy in a majority of patients is suggestive of lymphocytic thyroiditis. Anti-thyroid antibodies are elevated in the serum of all patients (Weintraub, Fein, Goldman).

II. Polypeptide Hormones

A. Biosynthesis of Glycoprotein Hormones

In a continuation of studies of cell-free translation of pituitary messenger RNA, it was shown by microsequencing that the pre-alpha obtained with mouse tumor and normal pituitary mRNA each possessed an initiating methionine. This was followed by an hydrophobic amino terminal extension ("signal") peptide containing methionine in positions 14 and 17. Mouse tumor and bovine pituitary pre-alpha both had leucines in positions 12, 15, 19 and 22. The tumor thus can serve as a model for pituitary TSH biosynthesis.

TSH-alpha precursors were also detected in translation of mouse tumor mRNA. From the changes observed in apparent molecular weight and immunoreactivity, it appears that the TSH-beta precursor has a conformation which is different from that of the mature subunit (Weintraub, Giudice).

The regulation of TSH biosynthesis was studied in primary cultures of dispersed mouse thyrotropic tumor cells and pituitary cells from normal and hypothyroid rats. TRH caused an increase in biosynthesis and secretion and the response was blunted by T_3 and by somatostatin. Addition of 60mM potassium also increased TSH production (Weintraub, Marshall).

HCG biosynthesis was studied in monolayer cell cultures of a cloned human choriocarcinoma (JEG-3). The progressive increase in apparent molecular weight appears to be due to progressive glycosylation, as also observed in TSH biosynthesis. The largest molecular forms of both alpha-subunit and HCG-beta are found in the medium, and appear to result from their preferential secretion immediately following their synthesis. It was also found that free alpha-subunit in serum from a variety of patients had a larger molecular size than the alpha-subunit obtained by dissociating HCG. This probably results from a greater glycosylation of the free alpha, and may account for its decreased ability to combine with HCG-beta to form the complete hormone (Rosen, Weintraub, Dean).

B. Ectopic Polypeptide Hormone Syndromes

Studies have continued on the prevalence of glycoprotein hormone subunits in the serum of patients with various types of malignant tumors. Alpha subunit levels exceeded the 95% confidence limit, in men and women, as follows: a) 11% and 16% in lung cancer, b) 32% and 18% in GI cancer, c) 50% and 12% in carcinoid, d) 61% and 38% in islet cell cancer. Corresponding elevations of HCG-beta were: a) 40% and 16%, b) 28% and 34%, c) 0% and 50%, d) 6% and 19%. The data indicate that alpha and CG-beta may be useful markers for certain types of malignant tumors. Elevations have also been observed in malignant gastrinomas (Zollinger-Ellison syndrome). The $beta_1$-glycoprotein of pregnancy (SP_1) has also been measured and is elevated in some patients with choriocarcinoma, teratoma, and embryonal carcinoma. Studies on the ectopic production of these polypeptides in cell

culture from various types of cancer are also being pursued (Rosen, Weintraub).

III. Steroid Hormone Secretion

The role of adenosine in the secretory response of adrenal and testicular cells has been studied further. By examining the effects of a number of adenosine analogs, it has been shown that the two sites corresponding to the purine and ribosyl moieties are involved in steroid secretion coupling (Wolff).

IV. Studies on Protein Structure

Clathrin is the principal protein of the "coated vesicle," a cell organelle responsible for the transport of various hormones and proteins into cells. The size of this vesicle is apparently determined by clathrin, which forms structures of similar size in the absence of cell lipid. To understand this phenomenon, the molecular properties of clathrin and its aggregation products have been studied. Clathrin is a very asymmetric molecule with about 50% alpha helical and beta structure. Studies are under way on the kinetics and products of clathrin aggregation (Edelhoch, Johnson, Pretorius, Nandi).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45000-12 CEB

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Thyroxine-Protein Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.-Y. Cheng	Research Chemist	CEB NIAMDD
	J. Robbins	Chief, Clin. Endo. Br.	CEB NIAMDD
	H. Edelhoch	Senior Chemist	CEB NIAMDD
OTHER:	S. Morrone	Guest Worker	CEB NIAMDD
	F. Erard	Visiting Fellow	CEB NIAMDD
	G. Irace	International Fellow	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Physiology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.4	1.9	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Thyroxine-binding globulin (TBG) has been deglycosylated with a mixture of glycosidases. The results indicate that the oligosaccharides of TBG, which contains 23% carbohydrate, are not involved in thyroid hormone binding or in the antigenicity of the protein. Electron spin resonance was used to study the topography of T_4-binding sites in the serum transport proteins by employing a series of "spin"-labeled thyroxine analogs. The binding site of serum albumin is deeper than that of either prealbumin or TBG. T_4 is less rigidly held in the TBG site despite a much higher binding constant.

PHS-6040
(Rev. 10-76)

Project Description:

Thyroxine-binding globulin (TBG), prepared from human serum by an improved purification method, was treated with a mixture of neuraminidase, β-galactosidase, α-mannosidase and β-N-acetylglucosaminidase which resulted in the removal of approximately 87% of saccharides. Purification by thyroxine-Sepharose affinity chromatography gave a homogeneous protein as shown by equilibrium sedimentation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acid and N-terminal sequence analysis indicated that the protein moiety was intact. Deglycosylation had no effect on the stoichiometry of the binding of L-thyroxine as shown by tryptophanyl fluorescence quenching and equilibrium dialysis at pH 8.6 and 25°C. However, the affinity constant for L-thyroxine was reduced from $1.6 \times 10^{9}M^{-1}$ to $0.58 \times 10^{9}M^{-1}$. Analysis of radioimmunoassay data revealed that deglycosylation resulted in a slight decrease of the affinity constant for anti-TBG antibody from $3.9 \times 10^{10}M^{-1}$ to $1.8 \times 10^{10}M^{-1}$. These results suggest that the polypeptide moiety, rather than the heterosaccharides, contains the antigenic determinants. Removal of the majority of the heterosaccharides of TBG has only a minor effect on its immunoreactivity and on the binding of thyroid hormone.

Although the topography of the thyroxine binding site in PA has been delineated by X-ray crystallography, little is known regarding the size and shape of the thyroxine binding sites in ALB and TBG. In the present study a series of spin-labeled thyroxine analogs were synthesized and employed as "molecular dipsticks" to probe the binding site. A specific electron spin resonance (ESR) signal is produced when the free rotation of the spin-labeled molecule is restricted and is proportional to the degree of immobilization.

ESR measurements indicated that the nitroxide group of N-3-[1-oxyl-2,2,5,5-tetramethylpyrrolinyl) carbamyl] thyroxine ethylester was strongly immobilized upon binding to PA ($2T_{11}$=66 Gauss) and ALB ($2T_{11}$=65 Gauss); however, it was less immobilized when bound to TBG ($2T_{11}$=46 Gauss). Furthermore, a spin-labeled derivative of dansylamide was also found to be rigidly bound to PA ($2T_{11}$=65 Gauss) and ALB ($2T_{11}$=70 Gauss), while no bound signal could be detected for TBG. These results suggested that the nitroxide radical in the binding site of TBG is less constrained than in the sites of ALB and PA. The introduction of one glycyl residue between the pyrrolinyl ring and the thyroxyl moiety caused an enhanced mobility of the nitroxide group bound to PA ($2T_{11}$=52 Gauss), while no significant change in the mobility was observed when bound to TBG ($2T_{11}$=46 Gauss) and ALB ($2T_{11}$=62 Gauss). This indicated that the thyroxine binding site in PA is wider at the entrance of the binding channel where the side chain is located. When two more methylene groups were inserted, the free radical exhibited greater freedom of movement in both PA and TBG, suggesting that binding sites in these two proteins have a

similar depth of approximately 20Å. However, in the case of ALB, the nitroxide radical was still immobilized when 5 methylene groups were bridged (overall length of this analog is 23Å). The results are consistent with the known funnel-like shape of the binding site in PA and indicate that the binding site in ALB is deepest.

Publications:

1. Robbins, J., Cheng, S.-Y., Gershengorn, M., Glinoer, D., Chanmann, H.J. and Edelhoch, H.: Thyroxine transport proteins of plasma. Molecular properties and biosynthesis. Rec. Prog. Horm. Res. 34: 477-519, 1978.

2. Irace, G. and Edelhoch, H.: Thyroxine-induced conformational changes in prealbumin. Biochemistry 17: 5729-5733, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45003-05 CEB

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Model Reactions for the Synthesis of Thyroxine Residues in Thyroglobulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Pommier	Visiting Associate	CEB NIAMDD
	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD
OTHER:	J. Nunez	Visiting Scientist	CEB NIAMDD

COOPERATING UNITS (if any)

Unité de Recherche sur la Glande Thyroide et la Regulation Hormonale, INSERM, Bicetre, France

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive

PHS-6040 (Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45004-08 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Structure of Polypeptide and Protein Hormones and Lipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Edelhoch	Senior Scientist	CEB NIAMDD
	M. Johnson	Staff Fellow	CEB NIAMDD
	H. T. Pretorius	Staff Fellow	CEB NIAMDD
	P. Nandi	Visiting Scientist	CEB NIAMDD
OTHER:	R. E. Lippoldt	Sr. Health Services Officer	CEB NIAMDD
	S. Formisano	Visiting Scientist	CEB NIAMDD
	S.-Y. Cheng	Research Chemist	CEB NIAMDD
	J. Kien	Staff Fellow	LHN NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Protein Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.6	5.4	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have isolated from human and bovine brains purified fractions of coated vesicles, derived from the coated pit region of the membrane. Clathrin, the major protein component of these vesicles, has been extracted from them and purified to apparent homogeneity. We have characterized by various hydrodynamic and spectroscopic techniques the clathrin particle which is extracted from the coated vesicles and its monomer subunit. In addition, we have reformed the so-called clathrin "basket" from the extracted and purified protein and characterized some of its hydrodynamic properties.

We have measured various optical parameters of rhodamine-labelled tri-iodothyronine and shown the formation of an intramolecular complex.

PHS-6040
(Rev. 10-76)

Project Description:

Fluorescent derivatives of hormones (and proteins) are being increasingly employed to follow their binding, localization and internalization by target cells and tissues. A new ultrasensitive method of detecting fluorescently labeled molecules has been used by Pastan and his collaborators to visualize the endocytosis of several hormones (and proteins) from the fluorescence of labeled rhodamine derivatives. Rhodamine labeled T_3 has been investigated in this way. We have evaluated the effect of T_3 on the fluorescence and other optical parameters of rhodamine in order to see the effects of the two chromophores on each other. We have observed large deviations in all of the properties of the rhodamine chromophore in T_3-rhodamine when compared with either the chromophore itself or the analog, alanyl-rhodamine. These deviations are observed only in aqueous solution at low temperature since they disappear in alcohol solution or in aqueous solution at high temperature. The results can be interpreted in terms of the existence of an intramolecular folded complex in water at low temperatures.

We have begun to study a protein, clathrin, which is the principal protein of the coated vesicle, a cellular organelle responsible for specific transport of various hormones and proteins. These vesicles, which have been isolated from numerous tissues, are formed from the coated pit regions of cellular membranes. In addition to the specific uptake of extracellular molecules, such as nerve growth factor, alpha-2-macroglobulin, and low density lipoprotein, a variety of biologic functions of coated vesicles has been suggested by recent experimental evidence (Geisow, M., Nature 227, 90-91). The size of the vesicles is apparently determined by clathrin, which forms structures of similar size in the absence of lipid. Such structures have been termed "empty vesicles" or "baskets." We have determined several molecular properties of these basket structures, and begun to dissect the nature of the forces which direct their assembly.

We have found an intermediate aggregate of clathrin and measured its size by gel filtration, analytical ultracentrifugation and light scattering. It is a very asymetric molecule, in contrast to the symmetric basket it can form. The circular dichroism spectrum of the intermediate aggregate suggests the presence of considerable alpha helical and beta pleated sheet structure: computer regression analysis assigns over half of the total number of amino acid residues to ordered structures. We have isolated the monomer of clathrin and determined its subunit molecular weight by equilibrium sedimentation in concentrated guanidine hydrochloride. An analytical value for the size of the assembled baskets has also been determined by velocity sedimentation coupled with measurement of the diffusion constant by autocorrelation using coherent light scattering (with the aid of Dr. Ralph Nossal of the computer services division).

Areas of present inquiry include the minimal number of proteins required for basket assembly, the kinetics of assembly, and isolation of their vesicular proteins, which may well include specific receptor proteins.

Publications:

1. Formisano, S., Johnson, M. L., George, L., Aloj, S. M., and Edelhoch, H.: Critical micelle concentrations of gangliosides. Biochemistry 18: 1119-1124, 1979.

2. Robbins, J., Cheng, S.-Y., Gershengorn, M. C., Glinoer, D., Cahnmann, H. J., and Edelhoch, H.: Thyroxine transport proteins in plasma. Molecular properties and biosynthesis. Recent Progress in Hormone Research 34: 477-517, 1978.

3. Edelhoch, H., and Robbins, J.: Thyroglobulin: Chemistry and Biosynthesis. In Werner, S. C. and Ingbar, S. H. (Eds.): The Thyroid. Harper and Row, New York, 1978, pp. 62-76.

4. Edelhoch, H.: Protein-lipid interactions and the role of water. In Quagliariello, E., Palmieri, F. and Singer, T. P. (Eds): Horizons Biochem. Biophys. Addison-Wesley Publishing Co., Reading, Massachusetts, 1978, Vol. 5, pp. 241-280.

5. Michot, J. L., Nunez, J., Johnson, M. L., Irace, G., and Edelhoch, H.: Iodide binding and regulation of lactoperoxidase activity toward thyroid goitrogens. J. Biol. Chem. 254: 1501-1505, 1979.

6. Alexander, S. S., Jr., Colonna, G., and Edelhoch, H.: The structure and stability of human plasma cold-insoluble globulin. J. Biol. Chem. 254: 1501-1505, 1979.

7. Irace, G., and Edelhoch, H.: Thyroxine-induced conformational changes in prealbumin. Biochemistry 17: 5729-5733, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45006-11 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Thyroid Iodoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. E. Burkhardt	Staff Fellow	CEB NIAMDD
	J. E. Rall	Director, Intramural Research Program	ODIPR NIAMDD
	M. Berman	Senior Scientist	LTB NCI

COOPERATING UNITS (if any)
National Cancer Institute

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Protein Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated

HHS-0040
(Rev. 10-76)

Publications:

1. Burkhardt, A. E., Berman, M., Haeberli, A., and Rall, J. E.: Synthesis of polypeptide chains of thyroglobulin. J. Biol. Chem. 254: 1476-1483, 1979.

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3. Rall, J. E.: The role of the laboratory in monitoring therapy and disease. In Young, D. S., Hicks, J., Nipper, H. and Uddin, D. (eds.): Clinician and Chemist. The Relationship of the Laboratory to the Physician. Washington, D.C. and Winston-Salem, N.C., The American Association for Clinical Chemistry, 1979, pp. 56-68.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-45009-12 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Studies in Thyroid Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Robbins	Chief, Clin. Endo. Br.	CEB NIAMDD
	B. D. Weintraub	Medical Director	CEB NIAMDD
	J. Goldman	Clinical Associate	CEB NIAMDD
	H. Fein	Clinical Associate	CEB NIAMDD
	M. Marshall	Clinical Associate	CEB NIAMDD
OTHER:	E. W. Chu	Head, Cytopath. Sec.	LP NCI
	M. Brennan	Head, Surg. Metab. Sec.	SB NCI
	A. E. Jones	Asst. Chief, Dept. of Nuc. Med.	CC NCI
	B. L. Weber	Dep. Chief, Surg. Path. and Post-mortem Sec.	LP NCI

COOPERATING UNITS (if any)
National Cancer Institute

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The method for radioiodine testing of functioning thyroid carcinoma was studied. The results show that performance of the whole-body scan two weeks after stopping T_3 provides a sufficient stimulus to detect a functioning thyroid remnant or metastasis. A study of serum TSH during the development of hypothyroidism in these patients showed a transient, marked increase of the α-subunit followed by increase in TSH-β and complete TSH. In some cases with the syndrome "inappropriate TSH secretion", there is inhibition of metabolism of thyroxine to reverse triiodothyronine. This appears to be a sensitive index of peripheral resistance to thyroid hormone. A new syndrome of autoimmune thyroiditis with transient hypothyroidism has been described. This occurs postpartum and evolves during a period of up to one year following delivery.

PHS-6040
(Rev. 10-76)

Project Descriptions:

The second phase of our study on the methods for radioiodine testing of functioning thyroid cancer is nearing completion. The findings indicate that a 2-week withdrawal of triiodothyronine therapy is sufficient to enable detection of almost all functioning lesions which can be found by a tracer test 2 weeks later, when the patients are usually severely hypothyroid. Adoption of the shorter withdrawal period should simplify significantly the follow-up of patients after thyroidectomy.

Thyroid Cancer Treatment

The dose of radioiodine used to ablate residual thyroid tissue after thyroidectomy is usually 50 to 100 mCi of ^{131}I. Following on reports in the literature, we are testing the adequacy of a 30 mCi dose, which can be administered to outpatients. Preliminary evidence suggests that this is a satisfactory dose, although a second or third treatment may be necessary to complete the thyroid ablation.

TSH Subunits in Hypothyroidism

We have noted the presence of very high concentrations of the α subunit of the glycoprotein hormones in certain patients with a recent onset of hypothyroidism. This elevation is out of proportion to the serum levels of hTSH or the TSH-β subunit. In order to study the dynamic relationship of the secretion of the subunits relative to hTSH, we have measured serum levels of subunits and TSH serially in athyreotic patients being withdrawn from triiodothyronine in preparation for whole body scanning. We have found the following: (1) As hypothyroidism develops the α subunit generally appears in serum before TSH or the β subunit; (2) the ratio of α subunit to TSH falls dramatically as hypothyroidism develops until it approaches the ratio noted in chronic hypothyroidism, and (3) the serum levels of α in acute hypothyroidism are higher than any described in a non-neoplastic condition except for uremia. These data suggest that, analogous to the temporal events in the developing fetus, during recovery from chronic TSH suppression the α subunit appears first while the β subunit is delayed and is limiting in TSH biosynthesis.

Inappropriate Secretion of TSH

We have continued our study of thyroid hormone metabolism in 2 patients with the syndrome of inappropriate TSH secretion. The disappearance half-time of administered ^{125}I-T_4 was normal in one patient, but prolonged to twice the normal value in another. The latter patient, with peripheral as well as pituitary resistance to T_4, had a markedly decreased serum rT_3 level, while the former patient with solely pituitary resistance had a moderately decreased serum rT_3. These studies suggest that altered T_4 metabolism may be a sensitive index of peripheral resistance, and could play a role in inappropriate TSH secretion. Studies are continuing to measure *in vivo* metabolism of

labeled rT_3 and T_3 in addition to T_4 in these and other patients with the syndrome. In addition, we plan to study the binding of thyroid hormones to nuclear receptors in circulating lymphocytes and cultured skin fibroblasts from these patients. Studies of immunoglobulin from these patients are described in Project No. Z01 AM 45023-03 CEB.

Postpartum Thyroiditis with Transient Hypothyroidism

We have identified five patients with a new syndrome of autoimmune thyroiditis with transient hypothyroidism. This entity had previously been described only in a single Japanese series that had ascribed it either to autoimmune or to subacute thyroiditis. The syndrome has a characteristic course: hyperthyroidism 2-3 months post-partum that may be asymptomatic and is associated with no neck symptoms, followed within 2-3 more months by hypothyroidism and goiter that then resolve before one year post-partum. Anti-thyroid antibodies have been elevated in all our patients; 4 of 5 have undergone fine needle aspiration biopsy and all have shown lymphocytic thyroiditis. We have begun studies of immune function and possible other autoimmune manifestations in these patients. The recognition of this syndrome has major implications for the routine evaluation of women in the post-partum period and we are beginning prospective studies to identify the prevalence of the syndrome in the Washington area.

Publications:

1. Larsen, P. R., Conard, R. A., Knudsen, K., Robbins, J., Wolff, J., Rall, J. E., and Dobyns, B. Thyroid hypofunctioning appearing as a delayed manifestation of accidental exposure to radioactive fallout in a Marshallese population. In Late Biological Effects of Ionizing Radiation, Vienna, International Atomic Energy Agency, Vol. 1, 1978, pp. 101-115.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45010-09 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Ectopic Protein and Subunit Production by Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. W. Rosen	Medical Director	CEB NIAMDD
	B. D. Weintraub	Medical Director	CEB NIAMDD
OTHER:	S. Aaronson	Lab Chief	LCMB NCI
	A. Rabson	Director, DCBD	OD NCI
	D. McCarthy	Sr. Investigator	DB NIAMDD
	J. Osborne	Sr. Investigator	IR MD NHLBI
	N. Javadpour	Sr. Investigator	SURG NCI
	M. Gail	Med. Stat. Res.	BB NCI
	M. Blackman	Instructor, Dept. Med.	Johns Hopkins U. Sch. Med.
	J. Kaminska	Ch., Radioimmunoassay Lab	Inst. Oncology, Warsaw
	L. Broder	Assistant Professor	Univ. Miami Sch. Med.
	E. Heyderman	Staff Scientist	Ludwig Inst., London
	R. Ruddon	Ch., Biol. Markers Lab	Frederick Cancer Res. Ctr.

COOPERATING UNITS (if any)

National Cancer Institute; Ludwig Institute, London, England; University of Miami

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	0.9	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We continue to study the sensitivity and specificity of placental proteins, chorionic gonadotropin and its free alpha (α) and beta subunits (CG-β), placental lactogen, and pregnancy-specific beta$_1$-glycoprotein (SP$_1$) as cancer markers in vivo and in vitro. Alpha and CG-β may be useful markers for certain malignancies, notably islet cell carcinomas of the pancreas. SP$_1$ is produced in vivo by certain non-seminomatous germ cell tumors of the testis. Ectopic production of SP$_1$ in vitro has been demonstrated in a number of malignant cell lines and was particularly high in a line derived from a fibrosarcoma.

PHS-6040
(Rev. 10-76)

Project Description:

A. In Vivo Studies

We have determined CG-β, α, LH-β, FSH-β and TSH-β in sera from a large number of cancer patients, normal persons, and patients with nonmalignant diseases.

Nonparametric statistical methods were used to establish sex-specific 50th, 90th, 95th and 99th percentile cutoff values for each assay in the major diagnostic subgroups. For men and women, respectively, prevalences of α values exceeding the 95% confidence limits of controls were a) 11% and 16% for lung cancer, b) 31.5% and 18% for GI cancer, c) 50% and 12.5% for carcinoid, and d) 61% and 38% for islet cell cancer. Corresponding elevations of CG-β were a) 40.5% and 16%, b) 28% and 34%, c) 0% and 50%, and d) 6% and 19%, respectively. Moreover, α "and/or" CG-β elevations occurred in a) 45% and 29%, b) 48% and 41%, c)50% and 50%, and d) 61% and 43%, respectively. In certain of these patients, elevated α "and/or" CG-β values were explained by elevated levels of CG or of eutopic LH or FSH. The data demonstrate that α and CG-β may be useful markers for certain malignancies. Furthermore, their eutopic secretion far exceeded that of pituitary β subunits. Thus, ectopic secretion of even closely related polypeptides is not a random process.

We determined serum α and CG-β in 23 patients with gastrinomas (Zollinger-Ellison syndrome). Among seven cases classified "malignant", α was elevated in 6 and CG-β was elevated in the seventh. Among 16 patients classified "benign" α remained normal in 10 (median followup, four years) but was elevated from the start in the other six cases despite clinical failure to detect metastases. Marked elevations of α occurred prior to death in three malignant cases. Alpha appears to be a useful marker for malignancy in the Zollinger-Ellison syndrome, although prolonged followup of the benign cases will be required to establish this.

SP_1, a recently identified $beta_1$-glycoprotein of pregnancy, has been determined in a radioimmunoassay in which a sensitivity of 0.1 ng/ml was achieved by use of an antiserum with $K_a \approx 1.7 \times 10^{11}$ L/mol and delayed addition of ^{125}I-SP_1 tracer. An additional anti-SP_1 serum, from an early bleeding, displayed "apparent" positive cooperativity in the IgG but not the IgM fraction. Using this radioimmunoassay we have determined SP_1 in sera of 97 men with germ cell tumors of the testis. SP_1 was elevated at 11-440 ng/ml in 3/6 with choriocarcinoma, 5/17 with teratoma or "teratocarcinoma" (embryonal carcinoma and teratoma) and 5/50 with embryonal carcinoma; the highest value in 143 patients with nonmalignant disease was 9.1 ng/ml. None of 24 sera from men with seminoma and none of 5 from men with orchitis had elevated SP_1. In the one case examined, testis choriocarcinoma SP_1 was similar immunochemically and by gel chromatography to placental SP_1.

B. In Vitro Studies

Assessment of ectopic protein production in vivo may be complicated by production of similar proteins from nonmalignant tissues. We examined SP_1 production in vitro by a number of cell lines derived from malignant human neoplasms: 18 carcinomas, 3 melanomas, 5 sarcomas, 3 astrocytomas, and 3 glioblastomas. Using a conservative criterion (>20% displacement in radioimmunoassay), nine lines (28%) produced SP_1. Production rates in pmol/mg cell protein/24 hr were 0.34-0.76 for three lines derived from bronchial carcinomas, 0.32, 0.36, and 0.79 for bladder, gall bladder and liver carcinomas, 0.58 for a glioblastoma, and 3.3 for kidney carcinoma. In all cases, much more SP_1 was secreted than retained. The highest production rate (33.6 pmol/mg/24 hr) was found in a line derived from a fibrosarcoma. Analysis of media and cell sonicates from this line by immunochemical dilution and by gel chromatography revealed that their SP_1 was similar to standard placental SP_1.

In contrast, none of the 32 lines examined produced detectable placental lactogen (<0.9 pmol/mg/24 hr). Ectopic production of chorionic gonadotropin and/or its beta subunit (0.5-5.6) was detected in 13 malignant lines (41%). Ectopic production of alpha subunit was detected in six malignant lines (9%); in two, derived from lung and liver carcinomas, alpha production attained rates of 391 and 506 pmol/mg/24 hr. Of the nine lines producing SP_1, two produced none of the other placental proteins examined, six also produced CG-β and one also produced alpha. Ectopic production of CG-β by the fibrosarcoma line was only 4% that of SP_1 production. This discordance of ectopically produced placental proteins is similar to that we have found in vitro and in vivo for the ectopic production of certain pituitary and carcinofetal proteins.

We studied the effects of butyrate and of dibutyryl cyclic AMP on the ectopic production of CG-β by a human glioblastoma line ("Coleman"). Previous workers, ourselves included, had found opposite effects of these agents on ectopic and eutopic production of CG and subunits; dbcAMP increased eutopic but had no effect on ectopic production, whereas butyrate did the reverse. In Coleman, however, ectopic production was markedly stimulated by dbcAMP whereas butyrate had very little effect.

Antisera purified by affinity chromatography are being developed to do immunocytochemical analysis of tissue sections from neoplasms producing placental proteins ectopically.

Preliminary analysis of highly purified SP_1, by techniques and ultracentrifugation of circular dichroism spectroscopy favor a homodimer with protomeric molecular weight of 44,700 and largely a beta pleated sheet configuration.

We have obtained a purified preparation of the most recently described placental protein, "PP_5". We are attempting to develop a radioimmunoassay of this glycoprotein.

Publications:

1. Broder, L. E., Rosen, S. W., Weintraub, B. D., Sussman, H. H., Primack, A., Hansen, H. H. and Muggia, F. M.: Placental proteins in bronchogenic carcinoma. In Rozencweig, M. and Muggia, F. (Eds.): Progress in Cancer Research and Therapy (Treatment of Lung Cancer), Vol. 11. New York, Raven Press, 1977, pp. 217-225.

2. Rosen, S. W. and Weintraub, B. D.: Ectopic placental lactogen. In Ruddon, R. W. (Ed.): Biological Markers of Neoplasia: Basic and Applied Aspects. New York, Elsevier, 1978, pp. 311-316.

3. Weintraub, B. D. and Rosen, S. W.: Ectopic subunits of glycoprotein hormones. In Ruddon, R. W. (Ed.): Biological Markers of Neoplasia: Basic and Applied Aspects. New York, Elsevier, 1978, pp. 327-334.

4. Broder, L. E., Weintraub, B. D. and Rosen, S. W.: Influence of chemotherapeutic agents on chorionic gonadotropin-alpha subunit secretion in a human lung cancer cell line (ChaGo): Discordance of cytotoxic and secretory effects. J. Nat. Cancer Inst. 61: 349-351, 1978.

5. Metz, S. A., Weintraub, B. D., Rosen, S. W., Singer, J. and Robertson, R. P.: Ectopic secretion of chorionic gonadotropin by a lung carcinoma: Pituitary gonadotropin and subunit secretion and prolonged chemotherapeutic remission. Amer. J. Med. 65: 325-333, 1978.

6. Tralka, T. S., Rosen, S. W., Weintraub, B. D., Lieblich, J. M., Engel, L. W., Wetzel, B. K., Kingsbury, E. W. and Rabson, A. S.: Ultrastructural concomitants of butyrate-enhanced ectopic alpha subunit and chorionic gonadotropin production in human bronchogenic carcinoma (ChaGo) cells. J. Nat. Cancer Inst. 62: 45-61, 1979.

7. Horne, C. H. W. and Rosen, S. W.: Human placental and pregnancy protein group report: Placental proteins in Nørgaard-Pedersen, B. and Axelsen, N. H.: Carcinoembryonic proteins. Recent progress with reviews by leading experts. Scand. J. Immunol. Suppl., 8, 55-64, 1978.

8. Rosen, S. W. and Weintraub, B. D.: Placental Lactogen and Other Hormones. In Herberman, R. B. and McIntire, K. R. (eds.): Immunodiagnosis of Cancer, Part I, Vol. 9, Marcel Dekker, Inc., New York and Basel, pp. 420-441, 1979.

9. Bender, R. A., Weintraub, B. D. and Rosen, S. W.: Prospective evaluation of two tumor-associated proteins in pancreatic adenocarcinoma. Cancer 43: 591-595. 1979.

10. Rosen, S. W. and Weintraub, B. D.: Chorionic gonadotropin. In Herberman, R. (Ed.): Compendium of Assays for Immunodiagnosis of Human Cancer. New York, Elsevier, pp. 63-66, 1979.

11. Rosen, S. W. and Weintraub, B. D.: Alpha subunit. In Herberman, R. (Ed.): Compendium of Assays for Immunodiagnosis. New York, Elsevier, pp. 59-62, 1979.

12. Rogol, A. D. and Rosen, S. W.: Heterogeneity of response of serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) to gonadotropin-releasing hormone (GnRH) in patients with the syndrome of congenital anosmia and hypogonadotropic hypogonadism. Proceedings of a Workshop on LH-RH testing. Memphis, Tenn., in press.

13. Cox, G. S., Weintraub, B. D., Rosen, S. W., Krutzsch, C., Stotler, I. and Maxwell, E. S.: High molecular weight forms of human placental lactogen (hPL): Synthesis in vitro and binding to membrane receptors for lactogenic hormones. Arch. Biochem. Biophys. 193: 355-367, 1979.

14. Rosen, S. W., Javadpour, N., Calvert, I. and Kaminska, J.: "Pregnancy-specific" Beta$_1$-glycoprotein (SP$_1$) is increased in certain nonseminomatous germ cell tumors. J. Natl. Cancer Inst. 62: 1439-1441, 1979.

15. Rosen, S. W., Kaminska, J., Calvert, I. and Aaronson, S.: Human fibroblasts produce "pregnancy-specific" beta$_1$-glycoprotein (PSßG, PAPP-C, SP$_1$) in vitro. Am. J. Obstet. Gynecol., in press.

16. Kaminska, J., Calvert, I. and Rosen, S. W.: A radioimmunoassay of SP$_1$ ("pregnancy-specific"-beta$_1$-glycoprotein). Clin. Chem. 25: 577-580, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45013-07 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Biosynthesis of Glycoprotein Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. D. Weintraub	Medical Director	CEB NIAMDD
	S. W. Rosen	Medical Director	CEB NIAMDD
OTHER:	B. S. Stannard	Physicist	CEB NIAMDD
	H. Fein	Clinical Associate	CEB NIAMDD
	M. C. Marshall	Clinical Associate	CEB NIAMDD
	D. Dean	Staff Fellow	CEB NIAMDD
	L. Giudice	Guest Worker	CEB NIAMDD
	M. J. Waxdal	Chief, Sec. on Mol. Structure	LI, NIAID

COOPERATING UNITS (if any)
NIAID

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.6	3.5	1.1

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have studied the biosynthesis of glycoprotein hormones in various normal and neoplastic cells derived from the pituitary and placenta. Cell-free translation of pituitary messenger RNA has demonstrated separate synthesis of a precursor form of TSH-α subunit (pre-α) and of TSH-β (pre-β). The pre-αs of three species have been microsequenced and found to have initiating methionine residues as well as complete homology in the position of leucines. Studies in intact thyrotrophs have demonstrated processing of pre-α initially to a form cleaved of the pre-piece and core-glycosylated, followed by addition of outer sugars necessary for combination with the limiting β subunit. Biosynthesis of TSH is stimulated by thyrotropin releasing factor and potassium and inhibited by thyroid hormones and somatostatin. Study of chorionic gonadotropin synthesis by choriocarcinoma cells has shown that only a small form of HCG-α accumulates in the cells while a processed large α form is selectively secreted along with complete HCG. Similar large forms of α are found in human sera and can be converted back to smaller forms by the selective removal of carbohydrate with mixed glycosidases.

HHS-0040
(Rev. 10-76)

Project Description

I. Biosynthesis of TSH

A. Cell-free translation of messenger (m)RNA

Poly (A) RNA from a mouse pituitary thyrotropic tumor and from normal mouse and bovine pituitaries was isolated and translated in two cell-free biosynthetic systems, a wheat germ extract and a rabbit reticulocyte lysate, in the presence of radiolabeled amino acids. Anti-bovine α serum immunoprecipitated precursor forms of tumor and pituitary α subunits ("pre-α's") from translations of their respective mRNA's. Pre-α subunits were analyzed by electrophoresis in SDS gradient polyacrylamide slab gels and the three pre-α's (mouse tumor, mouse pituitary and bovine pituitary) migrated identically, exhibiting an apparent molecular weight of approximately 17,000 daltons. Microsequencing of isolated mouse tumor and normal pituitary pre-α's showed that each possessed an initiating methionine followed by a hydrophobic amino terminal extension ("signal") peptide having the following partial amino acid sequences:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Mouse Pituitary	Met-	X-	X-	X-	X-	X-	X-	X-	X-	X-	X-	X-	X-	Met-	X-	X-	Met-	X-	X-	X-	X-	X-	X-
Mouse Tumor	Met-	X-	X-	X-	X-	X-	X-	X-	X-	X-	X-	Leu-	X-	Met-	Leu-	X-	Met-	X-	Leu-	X-	X-	Leu-	X-
Bovine Pituitary	Met-	X-	X-	X-	X-	X-	X-	X-	X-	X-	X-	Leu-	X-	X-	Leu-	X-	Leu-	X-	Leu-	X-	X-	Leu-	X-
Human Placenta*	Met-	X-	X-	X-	X-	X-	X-	Ala-	Ala-	X-	Phe-	Leu-	X-	X-	Leu-	X-	X-	Phe-	Leu-	X-	X-	Leu-	X-

*from Birken *et al, Biochem. Biophys. Res. Commun. 85* (1978) 1247-1253.

The results show that mouse tumor and pituitary pre-α subunits have Met in identical positions, supporting the premise that the tumor serves as a model of normal pituitary subunit biosynthesis. Furthermore, a strict conservation of the hydrophobic amino acid, Leu, was found in tumor and pituitary pre-α's and also in the homologous placental protein, pre-hCGα (Birken, et al., Biochem. Biophys. Res. Commun. 85 (1978), 1247-1253). The data suggest a common binding site in the rough endoplasmic reticulum for α subunits during

their biosynthesis either in the pituitary, the tumor, or the placenta. Studies are currently under way to determine the complete pre-sequence of mouse tumor and normal pituitary pre-α subunits from a number of species.

TSH-β subunit synthesis was detected in cell-free translations of mouse tumor mRNA in the absence of added microsomal membranes, only with an antiserum to the denatured form of the subunit. The molecular weight of the TSH-β-immunoreactive protein was estimated to be about 15,500, which is approximately 2,500 daltons greater than the protein moiety of authentic TSH-β. After processing of pre-TSH-β by microsomal membranes, the processed subunit was recognized only by an antiserum to the native subunit. Furthermore, antiserum to denatured TSH-β did not recognize TSH-β secreted by thyrotropic tumor cells in culture, whereas antinative TSH-β serum did. The results suggest TSH-β is synthesized in a precursor form whose conformation is different from that in membrane-processed TSH-β subunit or TSH-β in combination with α in the intact, secreted hormone.

B. Studies in dispersed thyrotroph cells

We have previously demonstrated precursor-product relationship in the biosynthesis of TSH in dispersed mouse pituitary tumor cells, using "pulse-chase" studies with [^{35}S]methionine. These studies had indicated that the alpha subunit is first recognized in intact cells as an 18,000 dalton form that has apparently been cleared of its pre-peptide and partially glycosylated. Subsequently, the 18,000 dalton α is processed to the "mature" 21,000 dalton form. Since the α subunit contains two asparagine-linked carbohydrate groups, we considered two possible mechanisms for this processing: (1) addition of the second "core" carbohydrate unit (GlcNac-GlcNac-(Man)n); (2) addition to both core units of the outer sugars (GlcNac-Gal-S.A.(Fucose)).

Using various [^{3}H]carbohydrates we studied the ratio of each in the 21,000 relative to the 18,000 dalton form, after normalizing the [^{3}H] counts to the ratio of [^{35}S]methionine in each peak. We observed that the ratio was about 1.0 for mannose but was 1.3-1.4 for N-acetyl glucosamine and galactose. These data support the second hypothesis of outer sugar addition. In addition, we observed an opposite ratio of 0.7 for fucose, consistent either with its unique addition to an inner core that is not processed as fully as non-fucosylated cores or with its removal during outer processing. There was no significant incorporation of [^{3}H]N-acetylmannosamine, a specific precursor of sialic acid, suggesting that mouse α, like bovine α, but unlike human α, is devoid of sialic acid.

Only the "mature" 21,000 dalton α combines with the limiting amounts of β subunit and is secreted. The 18,000 dalton TSH-β subunit that is just recognized in early pulses undergoes little subsequent processing. However, we have recently discovered an unusual "overglycosylated" form of TSH-β that is rich in glucosamine. Unlike TSH-α which can be secreted in a free form, TSH-β in the media is all combined with α as part of complete TSH.

C. Regulation of TSH biosynthesis

We have employed primary cultures of dispersed mouse thyrotropic tumor cells as well as cells from normal and hypothyroid rats to study regulation of TSH biosynthesis. Synthesized TSH was measured by immunoassay of secreted and intracellular TSH as well as by immunoprecipitation of [35S]-labeled TSH by antisera to bovine TSH-β and LH-α. In all three cell types 10 nM thyrotropin-releasing hormone (TRH) caused increases in total TSH (53-172%) and [^{35}S]labeled TSH (25-60%), as did 60 mM potassium (40-86% increases in total, and 8-25% increase in [^{35}S]TSH). Neither T_3 nor somatostatin (SS) caused a major inhibition of basal TSH synthesis within 24 hrs. However, the TRH-mediated response was blunted by both T_3 (15-35% decrease in total, 2-30% decrease in [^{35}S]TSH) and SS (25-39% decrease in total, 3-33% in [^{35}S]TSH) within 24 hrs.

These data suggest that T_3 and SS can inhibit stimulation of TSH biosynthesis; such an inhibition by hypothalamic SS would account for the high immunoactive/bioactive ratio of TRH in crude hypothalamic extracts.

II. Biosynthesis of HCG

A. Studies in a choriocarcinoma cell line

The clonal human choriocarcinoma cell line, JEG-3, secretes hCG and heterogeneous forms of its free alpha subunit. We have studied the relationship of these forms in *de novo* biosynthesis experiments. Cells at near confluence were labeled with [^{35}S]methionine by continuous (10 min to 24 hr exposure) and by pulse-chase (10 min exposure, 10 min to 3 hr chase) techniques. Media and cell lysates, chromatographed on Sephadex G-100, or, after reduction, electrophoresed on 12-20% gradient SDS-PAGE were analyzed by immunoprecipitation with antisera to the α and β subunit. The intracellular labeled free α subunit observed in lysates at 10 or 30 min continuous pulse was slightly smaller on G-100 (apparent mol. wt. 21,600) than urinary standard (22,700) but substantially smaller (15,100) on SDS-PAGE than urinary standard α (20,200). However, the predominant form of secreted free α subunit, at chase times as early as 30 min, migrated with a higher apparent molecular weight in both systems (SDS-PAGE = 22,200; G-100 = 29,300). We have not observed this secreted large free α subunit in the cell lysate but it appears to derive from processing of the small intracellular α followed by selective secretion. The β subunit of secreted hCG was larger (33,000) on SDS-PAGE than standard β (29,900). Secreted intact hCG migrated with urinary standard hCG on G-100. Analysis of [^{35}S]labeled intracellular hCG was originally complicated by co-precipitating large molecular weight proteins, but recent experimental modifications appear to have solved this problem. We are currently analyzing the intracellular processing of both hCG and α subunit.

B. Characterization of glycosylated forms of hCG and subunits

We have initiated studies relating to the chemical nature and

significance of heterogeneous forms of hCG and subunits. We studied the gel chromatographic behavior of native, denatured and partly deglycosylated hCG and subunits from a variety of sources: term placenta, pregnancy sera (first-third trimesters), purified urinary standards and sera from patients with ectopic hCG and/or free alpha production.

HCG and free alpha subunits in sera from all sources were larger than the corresponding forms within placental tissue or urinary standards. The free alpha subunits in sera were of similar size and were larger than alpha dissociated from hCG. After deglycosylation of hormones from all sources, the apparent molecular weights were markedly reduced but were not identical and new heterogeneous forms of ectopic alpha appeared. However, under denaturing conditions (that minimize carbohydrate effects) such differences (both before and after deglycosylation) were not observed.

We have concluded that extracellular hCG and free alpha contain more carbohydrate than intracellular forms and that the content of the ectopic glycoproteins differs from the eutopic. We have previously shown that free alpha subunit, both eutopic and ectopic, combines poorly with standard hCG-β. Experiments are in progress to determine if this apparent over-glycosylation of free alpha is responsible for its poor combination with beta, by studying the immunological (radioimmunoassay) and biologic (radioreceptor) activity of species produced by the combination of standard hCG-β with partially deglycosylated free alpha from these various sources.

Publications:

1. Kourides, I. A., Weintraub, B. D., and Maloof, F.: Large molecular weight TSH-β: The sole immunoactive form of TSH-β in certain human sera. J. Clin. Endocrinol. Metab. 47: 24-33, 1978.

2. Weintraub, B. D., and Stannard, B. S.: Precursor-product relationships in the biosynthesis and secretion of thyrotropin and its subunits by mouse thyrotropic tumor cells. FEBS Lett. 92: 303-307, 1978.

3. Kourides, I. A., Weintraub, B. D., Re, R. N., Ridgway, E. C., and Maloof, F.: Thyroid hormone, oestrogen, and glucocorticoid effects on two different pituitary glycoprotein hormone alpha subunit pools. Clin. Endocrinol. 9: 535-542, 1978.

4. Kourides, I. A., and Weintraub, B. D.: mRNA-directed biosynthesis of alpha subunit of thyrotropin: Translation in cell-free and whole-cell systems. Proc. Nat. Acad. Sci. (USA) 76: 298-302, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45014-08 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Adrenal Secretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Wolff	Medical Director	CEB NIAMDD
	G. H. Cook	Chemist	CEB NIAMDD
	C. Londos	Senior Staff Fellow	LNE NIAMDD
	D. M. F. Cooper	Visiting Fellow	LNE NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Purine-stringent (P) and ribose-stringent (R) sites for adenosine on adenylate cyclase have been further characterized. The further subdivision of the "R" site into a class responsible for cyclase stimulation has been distinguished from a class responsible for inhibition of the enzyme by the use of adenosine analogues.

PHS-6040
(Rev. 10-76)

Project Description:

The role of adenosine in secretory and other cellular responses occurring *via* adenylate cyclase has been further explored. Two sites responding to adenosine have been identified in various tissues. One is stringent for the purine moiety of adenosine, requires Mn^{++} for full expression, survives solubilization of adenylate cyclase, and is invariably inhibitory. The other is stringent for the ribose moiety (hence "R" site), is competitively inhibited by theophylline, tolerates modification in the purine moiety and usually stimulates adenylate cyclase (except in the fat cell). The R site can be subdivided into two subsets with the use of appropriate adenosine analogues that separate the stimulated site "Rs" from the inhibited site "Ri". These differences can also be demonstrated by analogue potencies for physiological responses such as steroid secretion in adrenal or Leydig cells or lipolysis in the fat cell.

Publication:

1. Londos, C., Wolff, J., and Cooper, D. M. F.: Actions of adenosine on adenylate cyclase. In Baer, H. P., and Drummond, G. I. (Eds.): Physiology and Regulatory Functions of Adenosine and Adenine Nucleosides. New York, Raven Press, 1979, pp. 301-309.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45015-09 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Thyroid Plasma Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Wolff	Medical Director	CEB NIAMDD
	A. R. Goldhammer	Staff Fellow	CEB NIAMDD
	G. H. Cook	Chemist	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.5	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Beef thyroid membrane adenylate cyclase activated with fluoride and then solubilized with Triton-N-101 shows activity at 120,000 daltons and a $\bar{V}$ = 0.75 (i.e., little detergent binding). The $\bar{V}$ is the same as for the Gpp(NH)p preactivated enzyme which, however, has a larger molecular weight (160,000).

PHS-6040
(Rev. 10-76)

Project Description:

Characterization of the adenylate cyclase of beef thyroid membranes continues. Previous molecular weight estimates by the H_2O/D_2O method yielded a value of 160,000 daltons for the activity solubilized from membranes preactivated with Gpp(NH)p. We now show that the fluoride-activated enzyme treated similarly has an apparent molecular weight of ∿120,000. This suggests the GTP-binding protein required for activation remained attached to the catalytic activity during solubilization with nonionic detergent as long as a G nucleotide was present but was not attached during fluoride-mediated activation.

Publication:

Asbury, R. F., Cook, G. H., and Wolff, J.: Soluble adenylate cyclase from thyroid membranes. J. Biol. Chem. 253: 5286-5292, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 45016-09 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Thyroid Hormone Secretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Wolff	Medical Director	CEB NIAMDD
	B. Rousset	Guest Worker	CEB NIAMDD
	S. Berkowitz	Staff Fellow	CEB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.9	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Adenosine and analogues modified in the ribose moiety inhibit TSH, Gpp(NH)p and F^--stimulated adenylate cyclase in beef thyroid membranes. The effect is markedly enhanced in the presence of Mn^{++} and is unaffected by theophylline. Since purine-modified analogues are inactive this response is characteristic of "P" site effectors in other tissues. We have also shown that lactoperoxidase binds to microtubules, oligomers and pure dimeric tubulin with stoichiometries ranging from 0.3 to 2.0. The affinity constant for tubulin is 1.10^6 M and the reaction seems to be specific with respect to related proteins. The binding is being explored as a purification method and for histochemical purposes.

PHS-6040
(Rev. 10-76)

Project Description:

Adenosine has both stimulatory and inhibitory effects on adenylate cyclase. Stimulation has not, so far, been demonstrable in beef thyroid membranes. Adenosine and certain adenosine analogues inhibit beef thyroid membrane adenylate cyclase. The inhibition has a rapid onset, is not directly on the catalytic or nucleotide regulatory sites, occurs with all activators tested (ITP, Gpp(NH)p, TSH, and F^-), and is seen also in mouse and human thyroid membranes. Addition of manganous ion, which activates adenylate cyclase, markedly enhances the inhibition by adenosine analogues. The order of potencies is: 2',5'-dideoxyadenosine>5'-deoxyadenosine>2'-deoxy-3'-phosphoadenosine>2'-deoxyadenosine>adenosine>adeninexyloside>adenine arabinoside. Purine-modified analogues are either inactive or stimulate slightly at high concentrations. This chemical specificity, the Mn^{++} requirement, and the lack of reversal by theophylline, suggest that these membranes have little "R" site activity (stringent for the ribose moiety) and primarily contain a "P" site that has stringent purine requirement but permits changes in the ribose moiety. This site appears to be associated with the catalytic unit since it persists in solubilized adenylate cyclase. Although numerous attempts have been made to locate a stimulatory effect in vivo (colloid droplet accumulation or McKenzie test) results to date have been difficult to reproduce.

A second aspect of our study of thyroid secretion involves the role of microtubules. In order to study tubulin interactions with thyroid organelles iodinated tubulin was prepared. However, it became apparent that iodinated tubulin lost its ability to copolymerize with native tubulin. Self-iodinated lactoperoxidase (LPO) did polymerize with tubulin through repeated cycles of polymerization/depolymerization to a constant specific activity and a stoichiometry of ~0.3 lactoperoxiase/tubulin dimer. Binding was decreased by low temperature, colchicine and high salt concentration. Since LPO is a basic protein (pI ~9.5), we investigated displacement of bound radioiodinated lactoperoxidase from microtubules by basic proteins. Neither ribonuclease A nor the basic isozyme of horseradish peroxidase caused significant displacement when unlabeled LPO displaced >80%. Serum albumin and the acidic isozyme of horseradish peroxidase were similarly inactive. Lactoperoxidase does not itself promote polymerization of pure tubulin. At very high concentrations, it prevents polymerization.

Lactoperoxidase also binds to the 30-36 S ring shaped oligomers of tubulin that are formed on depolymerization of microtubules but are stable to cold and colchicine. The stoichiometry LPO/tubulin dimer is 0.92. Apparently more binding domains are exposed in this structure than in microtubules. Finally, lactoperoxidase has been shown to bind to pure dimeric tubulin with an affinity constant of $\sim 1 \cdot 10\ M^{-1}$ but now with a stoichiometry approaching 2 LPO's per tubulin dimer. This binding is accompanied by a shift of the Soret spectrum and a large enough difference spectrum to permit assessment of binding. Binding appears to occur through at least one of the sulfhydryl groups of tubulin both on spectral grounds and because binding is prevented by sulfhydryl reagents. The molecular weight of the

2:1 LPO/tubulin complex is too small for the calculated minimum but is consistent with an association of 1 LPO with either the α or β subunit of tubulin. Presumably, the low molecular weight species consists of a mixture of LPO-α tubulin and LPO-β tubulin complexes. We are currently trying to obtain native subunits by this method. In addition, we have devised a purification method for tubulin using matrix-bound LPO and we plan to use the LPO binding to microtubules for localization studies in the electron microscope.

Publication:

1. Wolff, J., Londos, C., and Cook, G. H.: Adenosine interactions with thyroid adenylate cyclase. Arch. Biochem. Biophys. 191: 161-168, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45017-05 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (30 characters or less)
The Mechanism of Action of Lithium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. Wolff Medical Director CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive

PH-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45018-04 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Adenylate cyclase and Other Extracellular Products of *B. pertussis*: Effects on Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. L. Hewlett	Clinical Associate	CEB NIAMDD
	J. Wolff	Medical Director	CEB NIAMDD
OTHER:	L. Underhill	Physical Science Aid	CEB NIAMDD
	C. R. Manclark	Res. Microbiologist	FDA

COOPERATING UNITS (if any)
Division of Bacterial Products, Bureau of Biologics, FDA

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have identified and characterized in rabbit erythrocytes a protein activator of 105,000 daltons for adenylate cyclase of *Bordetella pertussis*. The protein is heat stable, does not activate by proteolysis and exhibits a lag period of 3-4 minutes for activation which is then only slowly reversible.

Project Description:

During attempts to study the effect of anti-pertussis sera on the adenylate cyclase activity of *B. pertussis*, both immune and preimmune sera were found to activate adenylate cyclase as a function of the degree of hemolysis. Moreover, organisms grown on blood agar had much higher cyclase activities than those grown on synthetic media. Search for an activator in erythrocytes yielded a protein of 105,000 daltons that was rapidly degraded to two smaller fragments. The activator exhibited a latent period of 3-4 minutes, was relatively heat stable and was not itself a protease even though activation was poorly reversible by washing of the organism. Preliminary evidence suggests that an additional cofactor is also required but has not yet been identified.

Publications:

1. Hewlett, E., Spiegel, A., Wolff, J., Aurbach, G., and Manclark, C. R.: *Bordetella pertussis* does not induce β-adrenergic blockage. Infection and Immunity 22: 430-434, 1978.

2. Hewlett, E. L., Underhill, L., Cook, G. H., Manclark, C. R., and Wolff, J.: A protein activator for the adenylate cyclase of *B. pertussis*. J. Biol. Chem. 254: 5602-5605, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45019-04 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Peroxidase Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Pommier	Visiting Associate	CEB NIAMDD
	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive

PHS-6040
(Rev. 10-76)

Publications:

1. Pommier, J., and Cahnmann, H. J.: Interaction of lactoperoxidase with thiols and diiodotyrosine. J. Biol. Chem. 254: 3006-3010, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45020-04 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Synthesis of Thyroxine Transport Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Goldman	Clinical Associate	CEB NIAMDD
	J. Robbins	Chief, Clin. Endo. Br.	CEB NIAMDD
OTHER:	R. McGuire	Research Biostatistician	VA Hosp., Wash., D.C.
	M. Berman	Senior Scientist	LTB NCI

COOPERATING UNITS (if any)
Veterans Administration Hospital, Washington, D.C.
National Cancer Institute

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive

PHS-6040
(Rev. 10-76)

Publications:

1. Glinoer, D., McGuire, R. A., Duboin, A., Cogan, J. P., Robbins, J., and Berman, M. Thyroxine-binding globulin metabolism in rhesus monkeys: Effects of hyper- and hypothyrodism. Endocrinology 104: 175-183, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45021-03 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Pituitary Gonadotropic Pathophysiology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. W. Rosen	Medical Director	CEB NIAMDD
OTHER:	J. M. Lieblich	Director, Med. Educ.	Highland Park Hosp., IL
	A. S. Rogol	Assoc. Prof. Pediatrics	Univ. Virg. Sch. of Med., VA
	B. White	Sr. Investigator	LEP NIAMDD
	K. Mittal	Research Microbiologist	BB DBBP
	J. Flier	Director, Diabetes Unit	Beth Israel Hosp., Boston, MA

COOPERATING UNITS (if any)
Highland Park Hospital, Dept. of Medicine, IL
Univ. of Virginia School of Medicine, VA
Diabetes Unit, Beth Israel Hospital, Boston, MA

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.1	0.4

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies continue on syndromes of hypogonadotropic hypogonadism with and without anosmia, and on patients with anosmia who are clinically eugonadal.

PHS-6040
(Rev. 10-76)

Project Description:

A. The Syndrome of Hypogonadism and Anosmia

Red cell phenotypes on the families of two men with hypogonadism and congenital anosmia were consistent with paternity by the patients ("fertile eunuchs"). In one case the red cell data were supported by HL-A typing and HL-A studies of the second man are in progress.

B. Familial Isolated Hypogonadotropic Hypogonadism

Two siblings with this syndrome were given subcutaneous LRF daily for 6 days. After this "priming", their response to intravenous LRF returned to normal.

C. Eugonadal Congenital Anosmia

Studies are in progress on a 24-year old man with congenital anosmia and an apparently normal pituitary-gonadal axis. We will attempt to determine whether he has subtle deficits in hypothalamic-pituitary function.

Publications:

1. Lieblich, J. M., Rosen, S. W., Guyda, H., Reardan, J., and Schaaf, M.: The syndrome of basal encephalocele and hypothalamic-pituitary dysfunction. Ann. Inter. Med. 89: 910-916, 1978.

2. Rosen, S. W., Gann, P., and Rogol, A. D.: Congenital anosmia: Detection threshholds for seven odorant classes in hypogonadal and eugonadal patients. Ann. Otol. Rhinol. Laryngol. 88: 288-292, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45022-03 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Regulation of Secretion of Hypothalamic Releasing Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. D. Weintraub	Medical Director	CEB NIAMDD
OTHER:	M. C. Marshall	Clinical Associate	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endorcinology Branch

SECTION
Endocrine Physiology

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Terminated

PHS-6040
(Rev. 10-76)

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45023-03 CEB

PERIOD COVERED

October 1, 1978 through September 30, 1979.

TITLE OF PROJECT (80 characters or less)

TSH Binding Sites: Relationship to Anti-thyroid Immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. D. Weintraub	Medical Director	CEB NIAMDD
OTHER:	F. Pekonen	Guest Worker	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Physiology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.2	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The nature of TSH-receptor interaction has been evaluated using whole thyroid cells, lymphocytes in continuous culture and thyroid membrane preparations. We have characterized two different TSH binding sites with different specificities and affinities. Only the high affinity site shows binding characteristics consistent with the receptor that mediates the biologic action of TSH. The latter site has been used to characterize the TSH receptor-binding activity of pituitary and placental glycoprotein hormones as well as immunoglobulins present in patients with various disorders of thyroid function.

PHS-6040
(Rev. 10-76)

Project Description:

We have characterized the binding of [^{125}I]bTSH to intact bovine and human thyroid cells and human lymphocytes, as well as to purified thyroid cell membranes. Our results suggest the existence of two different TSH binding sites on thyroid cell membranes, one with high affinity and specificity and one with lower affinity and less specificity. Incubation conditions affect the exposure of the different binding sites with predominantly high affinity, high specificity sites measured under close to physiologic incubation conditions and low affinity, low specificity sites measured under less physiologic conditions. We have further evaluated the carbohydrate nature of the high affinity, high specificity binding site by glycosidase treatment and have found that removal of sialic acid increases the number of such TSH binding sites. Removal of galactose, N-acetyl-glucosamine and fucose had little further effect on TSH binding, whereas exposure of thyroid membranes to a mixed glycosidase preparation decreased TSH binding.

By treating thyroid membranes with proteolysis inhibitors and strong salt solutions we have been able further to increase the number of high affinity TSH binding sites and have simultaneously increased the TSH sensitivity of the radioreceptor assay. With such methods we have compared the TSH receptor binding activity of different human pituitary (and placental) glycoprotein hormones as well as of immunoglobulins from patients with various disorders of thyroid function. We have found that human TSH preparations show binding activity correlated with their bioactivities, whereas FSH and LH have binding activities compatible with their reported contamination by TSH. Immunoglobulin preparations from patients with Graves' disease showed TSH-binding inhibitory activity in 80% of the cases with the strongest inhibitory activity by gamma globulins from patients with exophthalmos and pretibial myxedema. These results contrasted with the lack of TSH binding inhibitory activity of gamma globulins from patients with hyperthyroidism and inappropriate TSH secretion. Of the latter patient group only one out of 14 patients had TSH-binding inhibitory activity.

Recently we have developed a method for purification of TSH by immunoaffinity chromatography using agarose coupled to anti-TSH and anti-α. Purified TSH retained its immunoactivity, receptor-binding activity and adenylate cyclase-stimulating activity. We have further developed this method for separation of glycoprotein hormones and their α-subunit by elution of immunosorbent-bound material with linear guanidine gradients. With such immunoaffinity purification we have been able to compare the receptor-binding activity of serum and pituitary TSH.

We plan to use these methods for a general analysis of the biologic activity, receptor-binding activity and immunologic activity of heterogeneous forms of intracellular and extracellular TSH.

Publications:

1. Pekonen, F., and Weintraub, B. D.: Thyrotropin binding to cultured lymphocytes on thyroid cells. Endocrinology 103: 1668-1677, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45024-03 CEB

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Metabolism of Thyroid Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Sato	Visiting Associate	CEB NIAMDD
	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD
	J. Robbins	Chief, Clin. Endo. Br.	CEB NIAMDD
	J. Goldman	Clinical Associate	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Physiology

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.7	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The monodeiodination of thyroxine and related iodothyronines in monolayer cultures of monkey hepatocarcinoma cells was studied in relation to the cell growth cycle. Nonphenolic ring deiodination was markedly increased during the resting (G_1) phase, and phenolic ring deiodination was slightly increased. This change was due solely to an abrupt increase in enzyme synthesis.

PHS-6040
(Rev. 10-76)

Project Description:

The monodeiodination of thyroxine and related iodothyronines in monkey hepatocarcinoma cells has been studied in relation to the cell growth cycle. Cell division in the monolayer cultures ceases when the cells are grown in medium deficient in isoleucine and glutamine. Nonphenolic ring deiodination (e.g., conversion of T_4 to reverse T_3 or conversion of T_3 to 3,3'-T_2) is enhanced approximately 4-fold under these conditions. By the application of Lineweaver-Burk analysis in homogenates of the cells arrested in the G-1 growth phase, it has been demonstrated that the increased deiodination is the result of an increase in the amount of the enzyme rather than in the Km of the reaction. Phenolic ring deiodination (conversion of reverse T_3 to 3,3'-T_2) is affected in a similar fashion, but the increase in this enzymatic activity is much smaller. The quantity of the two deiodinases is changed to a different extent when a protein synthesis inhibitor is added to the arrested cells, indicating that two separate enzymes with different turnover rates are involved. Since the enzymatic assay was carried out in the presence of an excess of dithiothreitol, the changes cannot be the result of an alteration in sulfhydryl compounds.

Studies are underway to localize the nonphenolic ring deiodinase within the cell. Preliminary evidence indicates that it is located in the particulate fraction; but, in contrast to the phenolic ring deiodinating activity in normal rat kidney, it does not appear to be associated with the plasma membrane. Definitive evidence has not yet been obtained, however, since the normal enzymatic markers of plasma membranes in normal cells are markedly deficient in the hepatocarcinoma cells.

Publications:

1. Sorimachi, K. and Robbins, J.: Effects of propylthiouracil and methylmercaptoimidazole on metabolism of thyroid hormones by cultured monkey hepatocarcinoma cells. Hormone and Metabolic Research 11: 39-44, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45025-01 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

The effects of triiodothyronine on liver proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. E. Rall	Director, Intramural Research Programs	ODIRP	NIAMDD
	P. Vilkki	Visiting Scientist	CEB	NIAMDD
	J. M. Gavaret	Visiting Associate	CEB	NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Protein Structure

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.5	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two-dimensional isoelectric focusing and sodium dodecyl sulfate gel electrophoresis were adapted to investigate the number and kind of proteins affected by thyroid hormone. An acid protein 94kD pK 4.8 appeared in the liver of hypothyroid rats within 24 hours after administration of triiodothyronine. Several smaller proteins were increased at the same time.

PHS-6040
(Rev. 10-76)

The administration of thyroid hormones (TH) is associated with an increased level of many hepatic enzyme activities. A general growth-promoting activity of TH suggests that they may increase the synthesis of many liver proteins. To investigate this effect more directly liver proteins were analyzed by two-dimensional polyacrylamide gel electrofocusing. Hypothyroid rats were injected with triiodothyronine (T_3) (3 mg/100 g body weight) and the protein content of the different subcellular fractions of liver was analyzed 1, 2, 3 and 4 days after the injection. In the mitochrondrial fraction approximately 150 spots (molecular weights 17,000-180,000) can be easily and reproducibly identified when using ampholines (pH 5-7) in the isofocusing dimension. Three proteins (m.w. 55K, 68K and 130K and isoelectric points pH 6.0, 6.5 and 5.2, respectively) are increased and another protein, m.w. 94K isoelectric point pH 6.4, appears after treatment with T_3. These effects are seen 1 day after T_3 administration and are more pronounced after 4 days. A group of proteins of approximately 46kD and pH 6.4 is markedly decreased in the T_3-treated rats. A variety of acidic proteins (pK <5.0) not well separated by our present techniques also appears to increase after T_3. It is concluded that, of those proteins studied, the synthesis of a relatively few mitochondrial proteins is increased and the synthesis of another small number of proteins is decreased as a result of T_3 administration.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45026-01 CEB

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Model Reactions for the Synthesis of Thyroxine Residues in Thyroglobulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. M. Gavaret	Visiting Assoc.	CEB NIAMDD
	J. Pommier	Visiting Assoc.	CEB NIAMDD
	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD
	J. Nunez	Dir. of Res.	INSERM

COOPERATING UNITS (if any)

Unité de Recherche sur la Glande Thyroide et la Ré gulation Hormonale, INSERM, Bicetre, France

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The fate of the alanine side chain of the tyrosine in thyroglobulin that donates its aromatic ring to another diiodotyronine residue during thyroid hormone biosynthesis was investigated. It was found that, after hydrolysis of Tg, this side chain is present in the hydrolysate as pyruvic acid.

PHS-6040
(Rev. 10-76)

Project Description:

[U-^{14}C]Tyr-labeled Tg was synthesized in hog thyroid slices and purified by gel filtration. It was iodinated (with ^{127}I or ^{125}I) and then hydrolyzed by a combination of enzymes. The hydrolysate was fractionated on a column of the cation exchange resin AG50W-X4. Iodinations were carried out for various periods of time (0-90 min) and the elution profiles were compared.

With progressive iodination an acidic ^{14}C-labeled but iodine-free compound was formed concomitantly with thyroid hormone. The radioactivity ratio of this compound to hormone was 1:5. The acidic compound was identified by chromatographic and chemical methods as pyruvic acid. The precursor of the pyruvic acid in Tg may be dehydrolanine residues or pyruvoyl residues formed from dehydroalanine residues.

Publication:

1. Cahnmann, H. J., Pommier, J., and Nunez, J.: Spatial requirements for coupling of diiodotyronine residues to form thyroid hormone. Proc. Natl. Acad. Sci. USA 74: 5333-5335, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45027-01 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Synthesis of Thyroid Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Sato	Visiting Associate	CEB NIAMDD
	K. Sorimachi	Visiting Associate	CEB NIAMDD
	H. J. Cahnmann	Scientist Emeritus	CEB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Triiodo-L-thyronine (T_3) of high specific activity, labeled exclusively in the nonpleinolic (inner) ring has been synthesized by two iodination methods.

PHS-6040
(Rev. 10-76)

Project Description:

T_3 of high specific activity labeled exclusively in the nonpleinolic ring is required for metabolic studies, yet has not been available so far.

Two iodination procedures, using [3,5-^{125}I]diiodo-L-thyronine of high specific activity as the substrate have been worked out. One uses iodine in KI as the iodinating agent and the other iodide and chloramine T. The concentration of the iodinating agent as well as the pH of the reaction mixture are critical. Lower concentrations than the optimal ones give no or little T_3, while too high concentrations yield principally T_4. Also at optimal concentrations, the yield of T_3 is relatively low unless the pH of the reaction mixture is higher than the pK of the substrate. When the reaction is carried out at pH 11 a huge yeild of T_3 (70-75%) is obtained. No deiodination of the substrate or of the inner (labeled) ring of T_3 takes place during the iodination procedures.

Publication:

1. Sorimachi, K., and Cahnmann, H. J.: A simple synthesis of [3,5-^{125}I]-diiodo-L-thyronine and of [3,5-^{125}I]L-thyroxine of high specific activity: Endocrinology 101: 1276-1280, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 45028-01 CEB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Thyroid Hormones-Cell Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.-Y. Cheng	Research Chemist	CEB NIAMDD
	J. Robbins	Chief, Clin. Endo. Br.	CEB NIAMDD
OTHER:	I. Pastan	Chief, LMB	LMB, NCI
	M. Willingham	Senior Surgeon	LMB, NCI
	J. Maxfield	Visiting Fellow	LMB, NCI

COOPERATING UNITS (if any)
National Cancer Institute

LAB/BRANCH
Clinical Endocrinology Branch

SECTION
Endocrine Biochemistry

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.7	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to study the mechanism of thyroid hormones transport across the plasma membrane and intracellular distribution and localization in intact cells, fluorescent rhodamine-labeled thyroid hormones were synthesized. Rhodamine-labeled thyroxine (Rho-T_4) and triidothyronine (Rho-T_3) bind to the rat liver nuclear receptors with the dissociation constants of 70.8 nM and 20.3 nM, respectively. Studies with cultured GH_1 cells showed patches and caps on the surface after incubation of the cells with Rho-T_3 . No caps and patches were observed for rhodamine-labeled thyronine. Thus the interaction of Rho-T_4 and Rho-T_3 is specific. The physiological significance of these phenomena is currently under study.

PHS-6040
(Rev. 10-76)

Project Description:

In order to study the mechanism of the transport of thyroid hormones across the cell membrane and their intracellular distribution and localization in intact cells, hormones were synthesized. Rhodamine-labeled thyroxine (Rho-T_4) and 3,5,3'-triiodothyronine (Rho-T_3) bind to the solubilized rat liver nuclear receptor with the dissociation constant of 70.8 and 20.3 nM, respectively. The interaction of Rho-T_4 and Rho-T_3 with the nuclear receptor is specific, as the binding can be inhibited by T and rhodamine-thyronine (Tho-To) does not bind to the receptor protein.

Studies with cultured GH_1 cells indicated that Rho-T_3 enters the cells and fluorescence can be detected in the nucleus after incubation at 37°C for 0.5 hr in serum-free medium. Localization of Rho-T_3 in the nucleus is specific, as evidenced by failure to observe fluorescence in the nuclei after incubation of either the intact GH_1 cells or isolated nuclei with Rho-To. Furthermore, [^{125}I]-T binding to the nucleus or with the intact cells can be displaced completely by Rho-T_3 .

After incubation of GH_1 cells with Rho-T_3 at 37°C in serum-free medium, patches and caps of the fluorescent-labeled hormone on the plasma membrane were observed as early as 2 min. Their formation was specific for Rho-T_3 as only diffused fluorescence was seen when cells were incubated with Rho-To under identical conditions. The caps appeared to be on the outside of the membrane and attached to protein as they can be removed by trypsin. Formation of caps and patches was not observed in rat liver membrane under similar conditions.

After incubation of GH_1 cells with Rho-T_3 for 24 hrs at 37°C, fluorescent vesicles were observed in the cytoplasm. Whether these phenomena are related to the transport of thyroid hormones is currently under study.

Publication:

1. Cheng, S-Y., Eberhardt, N. L., Robbins, J., Baxter, J. D., and Pastan, I.: Fluorescent rhodamine-labeled thyroid hormone derivatives. FEBS Lett. 100: 113-116, 1979.

ANNUAL REPORT 1978 - 1979
Diabetes Branch, NIAMDD

INSULIN AND INSULIN RECEPTORS

Work in this area has been recognized by several awards (a) G. Burroughs Mider Award lectureship, (b) Berson-Yalow Award of the Society of Nuclear Medicine ($750.00), (c) Diaz Cristobal Prize to be presented at the triennial meeting of the International Diabetes Federation ($5,000.00), (d) Mosenthal Lectureship of the New York Diabetes Association, (e) an award to Pierre De Meyts from a Belgian Foundation for work he did at the National Institutes of Health called Prix des Alumni, and (f) grants from the Juvenile Diabetes Foundation, American Diabetes Association, Inc., and the Washington, D. C. Affiliate of the American Diabetes Association, Inc.

Receptors and Insulin in Brain and Other Organs: (Rosenzweig, Havrankova, Brownstein, Schmechel, and Roth) We had previously found typical insulin receptors in brain. In the present study, we showed that the insulin receptors in brain are unchanged under conditions in which receptors on liver or other peripheral tissues are elevated (insulin deficiency; starvation) or reduced (obesity; maturation).

Previously we had shown that the brain contains concentrations of insulin that are multi-fold higher than plasma and that this material is indistinguishable from genuine insulin by multiple criteria. In the present study we detected similar concentrations of insulin in liver and in essentially all other tissues (except possibly granulocytes and erythrocytes) in both rodents and humans. Again the cellular insulin is extremely similar to genuine insulin. Moreover, extreme hyperinsulinemia (obese mice) and hypoinsulinemia (streptozotocin-induced diabetes; starvation) produced little or no change in the concentration of insulin in brain, liver, or other sites. To explore the possible source of insulin, human transformed as well as normal cells were grown in tissue culture and were found to have insulin at concentrations similar to cells in vivo, despite the fact that the insulin concentration in the medium to which they were exposed was much lower. Furthermore, by differential radioimmunoassay, it was shown that the insulin in the tissue culture cells was not of bovine origin whereas the insulin in the medium was from bovine serum. These studies suggest the possibility that some, if not most of the insulin present in cells of brain, liver, and other tissues is synthesized by the individual cell. Studies are now under way to verify extra-pancreatic biosynthesis of insulin and to study the possible function of insulin in brain and at intracellular sites elsewhere.

Insulin Receptors in Humans: We recently devised a novel method to study receptors for insulin in the whole animal in vivo (in contrast to the usual methods which have permitted only the study of receptors on samples of tissue removed for in vitro study) (Wachslicht-Rodbard, Roth, McGuire, and Berman). During the past year we have extended the study to people and have shown its feasibility as a means of studying insulin receptors in disease states. This work was selected by the Society of Nuclear Medicine for the third Annual Berson-Yalow award.

The insulin receptors on blood cells of patients with a wide range of disorders in glucose metabolism have been studied by measuring ^{125}I-insulin binding to erythrocytes and/or to monocytes. Patients with anorexia nervosa were found to have elevated insulin binding that is corrected by refeeding. (Wachslicht-Rodbard, Gross, Rodbard, Ebert, and Roth). Patients with glucocorticoid excess were found to have a complex derangement of insulin binding to receptors on both erythrocytes and monocytes which in selected cases could be shown to be reversed with restoration of normal levels of glucocorticoids (Wachslicht-Rodbard, Gorden and Roth). In a series of studies in patients with lipoatrophic diabetes associated with severe insulin resistance it was found that insulin binding to receptors showed a broad scope of findings suggesting that multiple etiologies are probably responsible for this form of extreme insulin resistance (Wachslicht-Rodbard, Saviolakis, and Kahn). Preliminary studies were initiated to characterize insulin receptors in young infants by taking advantage of the insulin receptors on erythrocytes which permit studies of insulin receptors in very small quatities of blood (Wachslicht-Rodbard, Roth, Chrousos, and Cornblath).

Insulin Antibodies - Allergy and Resistance: (Kahn, Rosenthal, and Van Obberghen) Treatment of diabetics with insulin is followed uniformly by an immune response to the insulin (which is of animal origin). In only a small minority of the patients does the immune response produce untoward effects. A very large group of affected patients have been studied for both humoral and cellular immune characteristics in what is clearly by far the largest study of its kind and which updates almost two decades later the pioneer work of Berson and Yalow. In particular they characterized in detail the genetic basis for the immune response to insulin in people and have provided successful therapy for many of these patients.

Anti-receptor Antibodies: Antibodies directed against the receptor for insulin have been detected (in addition to patients with type B of extreme insulin resistance) in patients with ataxia telangiectasia and in patients with selective immune deficient syndromes, and the titer of antibodies is correlated with the severity of the insulin resistance (Harrison, Bar, Itin, and Underhill). In addition, anti-receptor antibodies have been detected in the New Zealand obese (NZO) mouse which is a model of adult type human diabetes, not only with regard to carbohydrate metabolism but possibly also to vascular complications. The genetics and autoimmune characteristics of this animal are under study (Harrison, Melez).

Antibodies have been used as a tool for purifying insulin receptors from human placenta. In addition, they have been used for the development of a radioimmunoassay which provides an additional independent method for detection of the receptor (Saviolakis, Flier, and Harrison). The combination of the new detection techniques as well as affinity chromatography on anti-receptor antibody columns and on columns of lectins that bind receptor have been used for the purification of the receptor from human placenta. Further, immunization of mice with partially purified receptor was used to immunize mice and to produce several hybridoma lines that produce monoclonal anti-receptor antibodies (Harrison, Itin, Underhill).

It has been previously shown that these antibodies, in addition to blocking the binding of insulin, mimic the actin of insulin on both cell surface and intracellular insulin-mediated processes (Kahn). These studies have been extended to show that the ability of the antibody to block insulin binding is fully retained in the monovalent antibody but the insulinomimetic properties are lost. This provides the first antagonist of insulin action at the level of the receptor that has ever been described and should be a powerful tool for further studies (Kahn, Baird).

A clinical enigma have been raised by the finding that the antibodies are both insulin blocking and insulinomimetic whereas the patients that harbor these antibodies in vivo are generally extremely resistant to insulin with severe hyperglycemia, and only rarely show hypoglycemia. In studies of 3T3-L1 fatty fibroblasts, it could be shown that the insulinomimetic property of the antibody is quite shortlived and that within 12-24 hours of exposure of the cells to the antibody the antibody produces desensitization of the insulin pathway, not at the level of receptor but at some step just beyond hormone binding to the receptor. This finding is based on the fact that the antibody treatment of the cell produced insensitivity to both insulin and antibody but did not alter the cells response to vitamin K_5 or to polyamines, two insulinomimetic agents that initiate their biological response at a site beyond hormone binding to the receptor (Kahn, Grunfeld, and Van Obberghen). An important corollary relates to information transferred to the cell. In theory, hormone and receptor require each other in order to activate the cell. The finding that the anti-receptor antibody is fully capable of mimicking insulin action suggests that the program for insulin action resides entirely within the receptor and that the sole function of insulin is to bring out this program for the receptor.

The development of the monovalent antibody as a specific inhibitor of insulin binding to the receptor for insulin has provided an important tool to solve other biological problems. For example, it is known that insulin and the insulin-like growth factors are structurally similar that they share the same spectrum of biological activities and cross-react with each other's receptors but that insulin is more potent metabolically than the growth factors whereas the growth factors are more potent than insulin as growth stimulators. By use of the monovalent antibodies which block the insulin receptors but do not affect the growth factor receptors it was shown that the metabolic properties of both insulin and of the insulin-like growth factors are mediated via the insulin receptor whereas the growth-promoting properties of both insulin and of growth factors are mediated via the growth factor receptor (King, Kahn, Nissley, and Rechler). These data help to clarify further the pathophysiology of hypoglycemia produced by hypersecretion of an insulin-like growth factor by extrapancreatic tumors as well as the complex syndrome associated with the hyperinsulinemia in infants of diabetic mothers. This may also explain many features of insulin and growth factor effects in tissue culture systems.

Purification and Characterization of the Insulin Receptor: Treatment of human cells with ^{125}I and lactoperoxidase resulted in the incorporation of radioactivity into membrane proteins that have the characteristics of the insulin receptor. These labeled proteins were specifically precipitated by anti-receptor antibody and the immunoprecipitation was inhibited by unlabeled insulin. The radioactive subunits were characterized by gel electrophoresis

and ranged in molecular weight from 40,000 to 100,000 and represent at least two distinct types of subunits (Lang, Kahn, Chrambach, and Harrison).

The insulin receptor in plasma membranes was subjected to radiation inactivation, a technique which permits the determination of the molecular weight of the biologically functional molecule. Extensive studies suggest that there is one subunit that regulates receptor affinity which is of high molecular weight (350,000) whereas the binding activity for insulin resides in a smaller molecule of about 90,000 molecular weight. Irradiation results in rapid inactivation of the high molecular weight affinity regulator and only slowly results in destruction of the insulin binding sites (Harmon, Kahn, Schlegel, and Kempner).

Cooperativity and Down-Regulation: The thermodynamic parameters for the reaction of insulin with the high affinity state of the insulin receptors was evaluated from equilibrium studies at multiple temperatures between 5° and 37°. The reaction is endothermic and entropically driven at low temperatures and exothermic and enthalpically driven at high temperatures, which is considered to be typical of hydrophobic reactions and supports the notion that the loss of non-polar surface residues during formation of the hormone receptor complex is an important driving force in the reaction (Waelbroeck, Van Obberghen, and De Meyts).

That a hormone can act to regulate the affinity of its own receptors or to regulate the concentration of its own receptors has been exploited for insulin and for growth hormone respectively to increase the sensitivity of the radio-receptor assay. Recent work established this as a general approach for competitive binding assays (Eastman, Lesniak, Gorden, Roth, and De Meyts).

Morphological Correlates of Ligand Binding to Receptors on Cells: (Gorden, Carpentier, Van Obberghen, Lesniak, Orci, Freychet, Le Cam, and Cohen) The interaction of insulin with its specific receptor sites on hepatocytes and lymphocytes has been studied in detail. Initially hormone binding takes place at the cell surface followed by transfer of the radioactivity from the cell surface to intracellular sites near the plasma membrane and preferentially localized to lysosomal structures. This process is time- and temperature dependent and is not only found for insulin but for many of the labeled ligands including other hormones and anti-receptor antibodies. These new techniques, introduced by these investigators, have expanded greatly our understanding of hormonal interactions with receptors on target cells.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,000 - 06 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Cooperative Mechanisms Regulating Receptor's Affinity for Insulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. Van Obberghen	Visiting Associate	DB, NIAMDD
	P. De Meyts	Research Fellow	Intern. Inst. of Cell., Belgium
	J. Roth	Medical Director	DB, NIAMDD
	M. Waelbroeck	Research Fellow	Intern. Inst. of Cell., Belgium

COOPERATING UNITS (if any)
International Institute of Cellular and Molecular Pathology, Brussels, Belgium

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.9	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The thermodynamic analysis of the reaction of insulin with its receptor on the cultured human lymphocytes suggest that this reaction is typical of a hydrophobic reaction and supports the concept that the loss of non-polar surface residues in the formation of the hormone-receptor complex is an important driving force of the reaction.

PHS-6040
(Rev. 10-76)

Project Description:

The binding of insulin to its receptors yields curvilinear Scatchard plots and accelerates its dissociation from the receptors. This phenomenon is consistent with negatively cooperative interactions among receptor sites. Studies with insulin analogues, which vary 100-fold in their affinity for the receptor and in their biological potency, have suggested that the negatively cooperative interactions are triggered by the binding to the receptor of a distinct domain of the insulin monomer surface. This domain comprises some of the 8 carboxy-terminal residues of the B-chain and the A_{21}-asparagine. Burying of this "cooperative site" in the dimerization of insulin leads to a loss of negative cooperativity (De Meyts, P., et al., Nature, 273: 504-509, 1978).

Blundell and coworkers have suggested that the so-called "hydrophobic reaction" plays an important role in the reaction with its receptor of insulin and other polypeptide hormones like glucagon (Sasalin, K., Dockerill, S., Adamiak, D. A., Tickle, I. J., and Blundell, T. L.: Nature 257: 751-757, 1975).

Indeed, the insulin monomer appears to be folded around an inside core of buried hydrophobic residues but there are also two patches of clustered hydrophobic residues on the surface of the monomer. The first hydrophobic domain is part of the putative receptor-binding region, constitutes most of the "cooperative site" and is involved in the monomer-monomer interactions in the insulin dimer whereas the second hydrophobic domain is involved in dimer-dimer interactions in the insulin hexamer. If hydrophobic effects play an important role in the formation of the insulin-receptor complex, it should be possible to demonstrate it by a thermodynamic analysis of the reaction. In the present work, we have attempted a thermodynamic analysis for the reaction of insulin with its receptor on the cultured human lymphocyte. Such a detailed analysis has not been performed in a hormone-receptor interaction so far, although some fragmentary information is available. In summary, the thermodynamic parameters for the reaction of insulin with the high affinity state of its cellular receptors have been evaluated from equilibrium studies at multiple temperatures between 5°C and 37°C. Both the enthalpy and entropy changes, due to the formation of the hormone-receptor complex, decrease markedly with temperature, corresponding to a large heat capacity change of -766 cal/(mol.deg.) at 25°C. The reaction is endothermic and entropically driven at low temperature and exothermic and enthalpically driven at high temperatures. This thermodynamic behavior is typical of a hydrophobic reaction and supports Blundell's concept that the loss of non-polar surface residues in the formation of the hormone-receptor complex is an important driving force of the reaction.

Publications:

1. Waelbroeck, M., Van Obberghen, E., and De Meyts, P.: Thermodynamics of the interaction of insulin with its receptor. J. Biol. Chem., in press.

Awards:

Diaz Cristobal Award - J. Roth and P. De Meyts received this award from Spain, which is given for outstanding publications in diabetes research in any scientific journal.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,002 - 05 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Insulin and Insulin Receptors in Diverse Species

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Muggeo	Visiting Scientist	DB, NIAMDD
Other:	E. Van Obberghen	Visiting Associate	DB, NIAMDD
	S. Falkmer	Professor of Med	Univ Omea, Sweden
	P. De Meyts	Research Fellow	Int Inst Cell and Mol Path
	C. R. Kahn	Senior Surgeon	DB, NIAMDD
	J. Roth	Medical Director	DB, NIAMDD

COOPERATING UNITS (if any)
Department of Medicine, University of Omea, Omea, Sweden; International Institute of Cellular and Molecular Pathology, Brussels, Belgium

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been discontinued.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,003 - 04 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Insulin Receptors and Insulin Responsiveness in 3T3-L1 Fatty Fibroblasts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. Grunfeld	Research Associate	DB, NIAMDD
	E. Van Obberghen	Visiting Associate	DB, NIAMDD
	C. R. Kahn	Senior Surgeon	DB, NIAMDD
Other:	P. M. Spooner	Staff Fellow	LNE, NIAMDD

COOPERATING UNITS (if any)

Laboratory of Nutrition and Endocrinology, NIAMDD

LAB/BRANCH
Diabetes Branch

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.6	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin receptors and responsiveness have been studied in undifferentiated and fatty 3T3-L1 fibroblasts. With differentiation, 3T3-L1 fibroblasts develop lipoprotein lipase. Insulin and insulin receptor antibody stimulate release of lipase from the cells as well as increases intracellular lipoprotein lipase activity. Antibody to the insulin receptor mimics insulin in the 3T3-L1 cells in that it stimulates glucose transport and oxidation as well as rendering these cells insulin resistant. Recovery of the cells from antibody-induced insulin resistance has also been studied.

PHS-6040
(Rev. 10-76)

Project Description:

3T3-L1 fibroblasts differentiate into a fatty phenotype which has insulin stimulated glucose transport, glucose oxidation, lipoprotein lipase and fatty acid and triglyceride synthesis, as well as insulin inhibited lipolysis. The use of an insulin-responsive cultured cell line has allowed us to explore the effects of antibody against the insulin receptor on a long-term insulin action, stimulation of lipoprotein lipase activity and to develop an in vitro model for the insulin resistance seen in patients with these antibodies.

With differentiation, 3T3-L1 cells develop lipoprotein lipase, the enzyme responsible for cleaving external triglyceride to permit uptake of fatty acids. Insulin stimulates both the acute release of lipase from the cells and an increase in intracellular lipoprotein lipase activity. The latter requires both RNA and protein synthesis, taking 24 hr to reach its maximum. We have shown that antibody against the insulin receptor also mimics this long-term insulin effect. The dose response curve for antibody is identical to that found for both inhibition of insulin binding and for the acute insulinominetic activity of the antibody. The onset of antibody stimulation of lipoprotein lipase activity is similar to that of insulin except that the antibody is decreasingly effective between 12 and 24 hours. Macromolecular synthesis (and presumably gene expression) is also required for activity of the antibody.

This study has several implications for the mechanism of insulin action. Since the antibody against the insulin receptor does not interact with the insulin binding sites on the nucleus, it is unlikely that such sites are involved in this action of insulin, which does require participation of the nucleus. Likewise, it has been proposed that uptake of insulin into the cell followed by activation and partial degradation may be required for insulin's long-term effects. It is unlikely that the antibody could be processed in a similar way. Thus, we conclude that the interaction of a ligand with the insulin receptor on the cell surface with generation of a second messanger accounts for the action of insulin, or that the receptor, once activated by ligand, contains all of the necessary information for hormone action.

Antibody against the insulin receptor stimulates glucose transport and oxidation when acutely added to 3T3-L1 cells. This requires bivalency as monovalent Fab preparations are inactive. Both monovalent and intact antibody inhibit insulin binding. The monovalent antibody also inhibits insulin action, shifting the insulin dose response curve to the right but not decreasing insulin's maximal activity. After prolonged exposure (6 hr) of 3T3-L1 cells to antibody in complete tissue culture media, the insulinomimetic activity of the antibody is lost and the maximal response to insulin is dramatically reduced, rendering the cells insulin resistant. No further reduction in insulin binding is seen. We call this process antibody induced desensitization. This also requires bivalency as it does not occur with the monovalent Fab. Glucose is required in the media; however, glucose does not seem to be merely serving as an energy source, as two hexoses, which do not serve as effective energy sources, 2-deoxyglucose and glucosamine can substitute for glucose.

Project Description Continued:

Recovery from antibody-induced insulin resistance has also been studied. Recovery of insulin's biological activity requires 6 hours for completion. Recovery of insulin binding is slower, and is only 40% complete at that time. We have previously shown that 3T3-L1 cells require only 40% of their receptors to be occupied in order to see the maximal response to insulin. Thus recovery is consistant with the number of spare receptors. Neither the development of or recovery from antibody-induced insulin resistance was blocked by chloroquine (an inhibitor of antibody degradation), cycloheximide (an inhibitor of protein synthesis), cytochalasin E (an inhibitor of microfilament function), or colchicine (an inhibitor of microtubule function). Studies with ^{125}I-labeled antibody indicate that recovery is consistant with the dissociation of antibody from the receptor and reversal of desensitization.

Publications:

1. Karlsson, F. A., Van Obberghen, E., Grunfeld, C., and Kahn, C. R.: Desensitization of the insulin receptor at an early post receptor step by prolonged exposure to anti-receptor antibody. Proc. Natl. Acad. Sci. (USA), 76: 809-813, 1979.

2. Karlsson, F. A., Grunfeld, C., Kahn, C. R., and Roth. J.: Regulation of insulin receptors and insulin responsiveness in 3T3-L1 fatty fibroblasts. Endocrinol. 104: 3183-3192, 1979.

3. Van Obberghen, E., Spooner, P. M., Kahn, C. R., Chernick, S. S., Garrison, M. M., Karlsson, F. A., and Grunfeld, C.: Antibodies against the insulin receptor elicit a late insulin effect: Increase in lipoprotein lipase in 3T3-L1 cells. Nature, 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,005 - 07 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Insulin Receptors in Normal Humans and in Patients with Insulin Resistance and Other Disease States

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Wachslicht-Rodbard	Visiting Associate	DB, NIAMDD
	G. A. Saviolakis	Clinical Associate	DB, NIAMDD
Other:	J. Roth	Medical Director	DB, NIAMDD
	P. Gorden	Medical Director	DB, NIAMDD
	C. R. Kahn	Senior Surgeon	DB, NIAMDD
	H. Gross	Clinical Associate	LCS, NIMH
	G. P. Chrousos	Clinical Associate	NPMB, NICHD
	M. Cornblath	Medical Director	IRP, NICHD

COOPERATING UNITS (if any)
Laboratory of Clinical Science, National Institute of Mental Health; Intramural Research, Division of Pediatrics, and Neonatal and Pediatric Medicine Branch, National Institute of Child Health and Human Development

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.8	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin binding to human erythrocytes has been evaluated in a number of different conditions that affect carbohydrate metabolism, in adults and in children. Such conditions include anorexia nervosa, lipoatrophic diabetes, hypercortisolism, glycogen storage diseases, nesidioblastosis, diabetes mellitus, insulin resistance and acanthosis nigricans (Type A and B), insulinomas, obesity. Insulin binding to monocytes was also evaluated in adults in many of the above listed diseases.

Project Number: Z01 AM 47,005 - 07 DB

Project Description:

Insulin binding to erythrocytes has been evaluated in patients with anorexia nervosa (eight females), in the basal cachectic state (seven patients) and after weight gain (eight patients). The values obtained were compared to those from 17 normal volunteers (eight females and nine males). Untreated patients showed increased insulin binding to receptors; after weight gain, these increased levels returned to normal. Increased binding was due to an increased number of receptors per cell, with little or no change in receptor affinity. In five patients, results of insulin binding to erythrocytes correlated closely with results obtained with monocytes. We concluded that in patients with anorexia nervosa, insulin binding to receptors is altered and that the abnormality is corrected by restoration of normal food intake and body weight.

In 11 patients with lipoatrophic diabetes and severe insulin resistance we have studied insulin receptors on erythrocytes and on monocytes. Nine patients were evaluated by insulin binding to monocytes, and three different patterns of receptor abnormalities were observed: decreased binding due to decreased binding capacity, normal tracer binding with decreased receptor affinity and normal or even increased binding. Insulin binding to erythrocytes in nine patients demonstrated similar heterogeneity of initial binding. In most cases there was a good correlation between the findings in monocytes and erythrocytes.

There was no obvious correlation between the nature of the receptor defect and the clinical patterns of these patients. Antibodies to the insulin receptor were not detected in any of these patients by either binding inhibition or immunoprecipitation.

Insulin binding to erythrocytes was also studied in normal infants and in infants with glycogen storage disease, nesidioblastosis and leucine sensitivity. Insulin binding in normal infants does not differ from normal adults. Nesidioblastosis and leucine sensitivity are characterized by elevated insulin binding. In glycogen storage disease insulin binding to erythrocytes is also elevated, except in one patient who had associated hepatic cirrhosis.

We are in the process of studying additional patients with diseases of altered insulin sensitivity and comparing the results obtained by the erythrocyte and monocyte methods.

Publication:

1. Wachslicht-Rodbard, H., Gross, H., Rodbard, D., Ebert, M., and Roth, J.: Increased insulin binding to erythrocytes in anorexia nervosa: Restoration to normal with refeeding. N. Engl. J. Med. 300: 882-887, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,006 - 04 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Study of Insulin Receptors In Vivo

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Wachslicht-Rodbard	Visiting Associate	DB, NIAMDD
Other:	J. Roth	Medical Director	DB, NIAMDD
	M. Berman	Physicist	LPP, NCI

COOPERATING UNITS (if any)
Laboratory of Theoretical Biology, National Cancer Institute

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The insulin receptor in vivo has been studied in the whole animal and a computer model which describes the insulin-insulin receptor interaction in vivo has been developed. We are continuing these studies in humans in both normal and obese subjects.

PHS-6040
(Rev. 10-76)

Project Description:

Previously rabbits had been used to study the insulin receptor *in vivo*; we have now adapted this method to studies in humans, utilizing ^{125}I-pork insulin (with high affinity for receptor) and ^{125}I-pork proinsulin (with low affinity for receptors). We have studied three normal volunteers and three obese patients. Plasma samples were analyzed for insulin and proinsulin content by TCA precipitation and antibody binding. The results obtained by these two methods were in good agreement. We calculated the apparent volume of distribution; it was 2-3 fold higher for insulin than for proinsulin. No significant differences were observed between the normals and the obese patients. We estimated that 44-72% of the insulin outside of the pancreatic β-cells is bound to receptors *in vivo*. We are planning to extend these studies to other disease states with altered insulin sensitivity.

The computer model, developed by Dr. Berman, describing the insulin binding *in vivo* in the rabbit will be evaluated in the studies in humans.

Publications:

1. Wachslicht-Rodbard, H., and Roth, J.: A new method for the measurement of the insulin receptor compartment *in vivo* in man: Comparison with *in vitro* methods. J. of Nuclear Medicine, 1979, in press.

Awards:

Third Berson-Yalow Award of the Society of Nuclear Medicine

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,007 - 04 DB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Antibodies to the Insulin Receptor: Studies in Disease States and Use as Probes of Insulin Receptor Function and Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. C. Harrison	Visiting Scientist	DB, NIAMDD
	G. Saviolakis	Research Associate	DB, NIAMDD
	J. Hedo	Guest Worker	DB, NIAMDD
	K. Melez	Clinical Associate	NMD, NICHHD
Other:	A. Itin	Grant Supported Tech Assist	DB, NIAMDD
	L. Underhill	Grant Supported Tech Assist	DB, NIAMDD

COOPERATING UNITS (if any)

Neonatal Medicine Branch, National Institute of Child Health and Human Development, NIH

LAB/BRANCH

Diabetes Branch

SECTION

Receptors and Hormone Action Section

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	2.5	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The insulin receptor has been purified using a combination of wheat germ and receptor antibody affinity chromatography. The receptor antibodies have been used in a new radioimmunoassay for insulin receptors, which is being applied to study receptor phylogeny and function. Lectins are being used as tools to probe receptor function and to purify receptors. Immunoprecipitation of receptors has been used as an assay to screen for receptor antibodies. Low titer IgM antibodies have been found in patients with ataxia-telangiectasia and in the New Zealand obese (NZO) mouse - a model of human adult-type diabetes.

-6040
Rev. 10-7

Project Description:

The interaction of insulin receptor antibodies with the solubilized insulin receptor has been studied in detail. Solubilized insulin receptors were prepared by extracting human placental membranes with Triton-X-100, and assaying for ^{125}I-insulin binding activity using polyethylene glycol to precipitate receptors. These solubilized receptors retain all the properties, including negative cooperativity, of membrane-bound receptors. Receptor antibodies inhibited insulin binding to the solubilized receptors with the same titers as previously reported using whole cells. This inhibition was due to a decrease in the number of available insulin binding sites, rather than a decrease in their affinity as seen with membrane-bound receptors.

Immunoprecipitation of the Insulin Receptor:

^{125}I-insulin-labeled receptor could be immunoprecipitated by receptor antibodies after the addition of the second antibody (using anti-human IgG) or protein-A-Sepharose. The procedure was highly specific, resulting in selective extraction of only three major membrane proteins, as seen on polyacrylamide gels. Immunoprecipitation was a more sensitive assay for receptor antibodies than the standard binding inhibition assay. It enables us to detect antibodies against any determinant on the receptor, not only those which happen to impair insulin binding or evoke insulin-like effects.

Radioimmunoassay of the Insulin Receptor:

The specificity of the antibodies for precipitating the receptor has made them a useful reagent for measurement of the receptor in a classic radioimmunoassay procedure. Receptor is labeled with ^{125}I-insulin and incubated together with a fixed amount of antibody and increasing amounts of unlabeled receptor. Bound and free labeled receptor were separated by addition of a second antibody. Addition of a minimum of 10^{-10} M unlabeled insulin binding sites results in a decrease in the bound labeled receptor. This assay enables us, for the first time, to measure the receptor independent of its ability to bind insulin. We have demonstrated that receptors from different tissues and species are immunologically different and are applying the radioimmunoassay to the study of receptor ontogeny and phylogeny.

Affinity Purification of the Insulin Receptor:

The receptor antibodies have provided a unique means of purifying the insulin receptor. Previous attempts to purify the receptor by insulin-agarose affinity chromatography have resulted in very limited purification and very low recovery. However, a combination of wheat germ and receptor antibody affinity chromatography resulted in a receptor which was minimally 25% pure. It resolved on SDS-PAGE as one major component of mol. wt. 82,000 daltons and three minor components of 67,000, 55,000, and 35,000 daltons.

Project Description Continued:

The purified receptor was also resolved on a tailored Triton-polyacrylamide gel system into at least three insulin-binding components. When the major component was eluted and re-run on SDS-PAGE it had an apparent mol. wt. of 82,000. Efforts are currently being made to obtain sufficient quantities of purified receptor for chemical and structural analysis.

Six lectins have been identified which bind to the insulin receptor. Lectin treatment of solubilized membranes is being investigated as an alternative procedure for receptor purification.

Delineation of Separate but Related Receptors for Insulin and MSA Growth Factor in the Human Placenta:

Human placental membranes, in both particulate and Triton-solubilized forms, were shown to have functionally-distinct binding sites for insulin and multiplication-stimulating activity (MSA). Autoantibodies to the insulin receptor specifically precipitated the insulin, but not the MSA, receptor.

Production of Monoclonal Antibodies to the Insulin Receptor:

Partially-purified human placental insulin receptors were used to immunize Balb/C mice. After 4 days the mice spleen cells were hybridized to the P3 X 63 line of mouse myeloma cells (kindly donated by Dr. G. Eisenbarth). Several hybridoma lines grew out which appeared by several criteria to be secreting receptor antibodies. These have been passaged as ascites tumors in Balb/C mice prior to cloning.

NZO mice sera were found to contain low titer (1/4 - 1/32) IgM antibodies to the insulin receptor, which inhibited insulin binding and immunoprecipitated the receptor. The pathogenetic role of these antibodies in the insulin-resistant diabetes of these obese mice is being investigated.

Breeding studies conducted by Dr. Katalin Melez on NZO mice, to the F_2 back-cross state, indicate that the metabolic syndrome (identified by hyper-insulinemia) is probably inhertied as a sex-linked autosomal recessive trait. The spectrum of autoimmunity in the NZO mouse has been enlarged to include antibodies to single and double-stranded DNA, and immunoglobulin (predominantly IgM) deposition in the kidneys. The kidney lesions include basement membrane thickening, thickening of the afferent and efferent arterioles, mesangial desposition of nodular PAS + material resembling the Kimmelsteil-Wilson nodules of human diabetes, and cellular infiltration. The findings indicate that the NZO mouse can be used to study immunological mechanisms causing diabetes and its vascular complications.

Publications:

1. Harrison, L. C., Flier, J. S., Roth, J., Karlsson, F. A., and Kahn, C. R: Immunoprecipitation of the insulin receptor: A sensitive assay for receptor antibodies and a specific technique for receptor purification. J. Clin. Endocrinol. Metab. 48: 59-65, 1979.

2. Harrison, L. C., Flier, J. S., Itin, A., Kahn, C. R., and Roth, J.: Radioimmunoassay of the insulin receptor; New probe of receptor structure and function. Science 203: 544-547, 1979.

3. Harrison, L. C. and Itin, A.: A possible mechanism for insulin resistance to the insulin receptor. Nature 279: 334-336, 1979.

4. Harrison, L. C., Flier, J. S., Kahn, C. R., and Roth, J.: Autoantibodies to the insulin receptor: Clinical and molecular aspects. in Autoimmune Disease (N. R. Rose, P. E. Bigazzi and N. L. Warner, eds.) Elsevier-North Holland, New York 1978, 61-76.

5. Harrison, L. C., and Kahn, C. R.: Autoantibodies to the insulin receptor. Prog. Clin. Immuno. Vol. 4, in press.

6. Harrison, L. C.: Insulin receptor autoantibodies: Clinical and molecular aspects. Circulation Research, in press.

7. Bar, R. S., Levis, W. R., Rechler, M. M., Harrison, L. C., Siebert, C. W., Podskalny, J. M., Roth, J., and Muggeo, M.: Extreme insulin resistance in ataxia telangiectasia: Defect in the affinity of insulin receptors. New Engl. J. Med. 21: 1164-1171, 1978.

8. Bar, R. S., Harrison, L. C., Muggeo, M., Gorden, P., Kahn, C. R., and Roth, J.: Regulation of insulin receptors in normal and abnormal physiology in humans. Adv. Horm. Res. 24: 23-52, 1979.

9. Muggeo, M., Bar, R. S., Harrison, L. C., Roth, J., and Kahn, C. R.: Pathophysiology of insulin receptors in man. in Obesity in Childhood (E. Caciari, S. Raiti, and C. Laron, eds.), Academic Press, London, 1979.

10. Bar, R. S., Harrison, L. C., Roth, J., Kahn, C. R., and Muggeo, M.: Insulin Receptors in obesity. in Obesity in Childhood (E. Cacciari, S. Raiti, and C. Laron, eds.), Academic Press, London, 1979.

11. Harrison, L. C.: Current concepts: Insulin resistance in obese diabetic patients. Consultant 19: 64-69, 1979.

12. Harrison, L. C., and Flier, J. S.: Diabetes associated with other endocrine diseases. in Secondary Diabetes: The Spectrum of the Diabetic Syndrome (S. Podolsky and M. Viswanahan, eds.), Raven Press, New York, in press.

Publications Continued:

13. Kahn, C. R., Flier, J. S., Muggeo, M., and Harrison, L. C.: Autoantibodies to the insulin receptor in insulin resistant diabetes. in The Immunology of Diabetes (W. J. Irvine, ed), Macmillan, London, in press.

14. Harrison, L. C., Flier, J. S., Kahn, C. R., and Roth, J.: Autoantibodies to the insulin receptor. Prog. Clin. Immuno., in press.

15. Harrison, L. C., and Roth, J.: The pathophysiology of peptide hormone receptors. Aust. New Zeal. J. Med., in press.

Awards:

A. I. is supported by a grant from the Juvenile Diabetes Foundation.
L. U. is supported by a grant from the American Diabetes Association, Inc.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,008 - 04 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Detection and Characterization of Cell Surface Receptors Using Specific ^{125}I-Receptor Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. B. Jarrett	Guest Worker	DB, NIAMDD
	E. Van Obberghen	Visiting Associate	DB, NIAMDD
Other:	J. Roth	Medical Director	DB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been discontinued.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,009 - 04 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Anti-receptor Antibodies as Probes of Insulin Action

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. R. Kahn	Senior Surgeon	DB, NIAMDD
	G. L. King	Research Associate	DB, NIAMDD
	K. L. Baird	Bio Lab Tech	DB, NIAMDD
Other:	M. M. Rechler	Senior Surgeon	LBP, NIAMDD
	S. P. Nissley	Senior Investigator	MET, NCI
	J. Larner	Prof of Biochemistry	Univ of Virginia, Charlottesville

COOPERATING UNITS (if any)
Laboratory of Biochemical Pathology, NIAMDD; Metabolism Branch, National Cancer Institute; Department of Biochemistry, University of Virginia, Charlottesville, Virginia

LAB/BRANCH
Diabetes Branch

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sera which contain anti-insulin receptor antibodies were used as probes of insulin receptors in rat adipocytes, human fibroblasts, and rat muscle. These sera block insulin binding, and acute exposure had insulin-like biological effects. This activity resided in the $F(ab'_2)$ portion of the molecule. Fab' fragments bind to receptor, block insulin binding and action, but are without bioactivity. These findings suggest an important role for receptor aggregation in insulin action. The Fab' also provides the first competitive inhibitor of insulin action at the receptor level.

OD 1-60-40
(Rev. 10-76)

Project Description:

Exposure of adipocytes to antibodies to the insulin receptor results in a blockade of ^{125}I-insulin binding, stimulation of glucose oxidation, and multiple other insulin-like effects, including activation of the intracellular enzyme glycogen synthase and inhibition of phosphorylase. Allowing for differences in purity, anti-receptor antibody is equipotent with insulin on a molar basis. Both the bivalent $F(ab')_2$ fragment and monovalent Fab' of the anti-receptor antibody are fully active in inhibiting ^{125}I-insulin binding. Bivalent $F(ab')_2$ also retains its insulin-like effects. In contrast, the monovalent Fab' loses all ability to stimulate glucose oxidation. Addition of anti-Fab_2 anti-sera which cross-link the Fab'-receptor complexes results in a restoration of the insulin-like activity of the antibody. Similarly, if cells are exposed to submaximal doses of insulin, addition of anti-insulin antibodies in low concentration enhances the biological activity of insulin. These data suggest that receptor occupancy by ligand is not sufficient for signal generation and that the insulin-like effects of anti-receptor antibody (and perhaps insulin itself) require receptor aggregation or clustering.

Insulin and the insulin-like growth factors, such as MSA (Multiplication Stimulating Activity), are chemically related polypeptides that have common biological activities and cross-react with each other's specific receptors. Both insulin and MSA produce acute metabolic responses (glucose oxidation in adipocytes) and chronic growth responses (DNA synthesis in cultured fibroblasts). To determine whether these effects are mediated by insulin receptors, MSA receptors, or both, we have selectively blocked insulin receptors with a pure insulin receptor antagonist, namely, Fab fragments derived from naturally-occurring antibodies to the insulin receptor. In adipocytes 10 µg/ml of Fab inhibited insulin binding by 90% whereas MSA binding was inhibited by only 5%. This blockade of insulin receptors produced a 30-fold shift in the dose response for stimulation of glucose oxidation by both insulin and MSA, suggesting that the acute metabolic effect of both peptides is mediated via the insulin receptor. In cultured human fibroblasts, 10 µg/ml of Fab inhibited insulin binding by 90% and MSA binding by 15%. In this case, however, blockade of the insulin receptor did not alter the dose response for stimulation of thymidine incorporation into DNA by either insulin or MSA. Furthermore, intact anti-receptor antibody which produces multiple other insulin-like effects did not stimulate thymidine incorporation by itself. These data demonstrate directly that insulin and MSA mediate their metabolic effects via interaction with the insulin receptor, whereas the growth-promoting action of both peptides is mediated via the receptor for MSA and other growth factors.

Publications:

1. Kahn, C. R., Baird, K. L., Jarrett, D. B., and Flier, J.S.: Direct demonstration that receptor cross-linking or aggregation is important in insulin action. Proc. Natl. Acad. Sci., USA, 75:4209-4213, 1978.

Publications Continued:

2. Lawrence, J. C., Jr., Larner, J., Kahn, C. R., and Roth, J.: Antibodies to the insulin receptor stimulate glycogen synthase in rat adipocytes. Mol. Cell Biochem. 22: 153-158, 1978.

3. Kahn, C. R. and Baird, K. L.: The role of receptor aggregation in insulin action. In Physical Chemical Aspects of Cell Surface Events in Cellular Regulation (ed.) C. DeLiss and R. Blumenthal, Elsevier-North Holland Publ. Co., New York, 1978, pp. 119-127.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,010 - 07 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

NSILA-s and Related Peptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. R. Kahn	Senior Surgeon	DB, NIAMDD
	C. M. Hendricks	Bio Lab Tech	DB, NIAMDD
	P. Gorden	Medical Director	DB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION
Clinical and Cellular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

NSILA-s is an insulin-like peptide which reacts with its own receptor and the insulin receptor. NSILA-s is elevated in about 40% of patients with hypoglycemia and non-islet cell tumors. NSILA-s is low in hypopituitary loses of growth hormone (GH), however, NSILA-s does not appear to respond acutely to changes in endogenous GH.

This project is temporarily inactive.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,013 - 07 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Radioreceptor Assay of Growth Hormone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. C. Eastman	Investigator	Maine Medical Center, Portland
	M. A. Lesniak	Research Chemist	DB, NIAMDD
Other:	P. Gorden	Medical Director	DB, NIAMDD
	J. Roth	Medical Director	DB, NIAMDD
	P. De Meyts	Research Fellow	Intern. Inst. of Cell., Belgium

COOPERATING UNITS (if any)
Maine Medical Center (Department of Internal Medicine), Portland, Maine
International Institute of Cellular and Molecular Pathology, Brussels, Belgium

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In hGH radioreceptor assay unlabeled hGH competes with ^{125}I-hGH for binding a fixed number of receptor sites on cultured human lymphocytes (IM-9). The ability of hGH to regulate its receptor concentration has been exploited to increase the sensitivity of the hGH in radioreceptor assay.

This project has been temporarily discontinued.

Rev. 10-76

Project Number: Z01 AM 47,013 - 07 DB

Project Description:

None

Publications:

1. Eastman, R. C., Lesniak, M. A., Roth, J., De Meyts, P., and Gorden, P.: Regulation of receptor by homologous hormone enhances sensitivity and broadens scope of radioreceptor assay for hGH. J. Clin. Endocrinol. Metab., 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,014 - 11 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Acromegaly

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Roth	Medical Director	DB, NIAMDD
Other:	P. Gorden	Medical Director	DB, NIAMDD
	R. C. Eastman	Investigator	Maine Medical Center, Portland

COOPERATING UNITS (if any)
Maine Medical Center (Department of Internal Medicine), Portland, Maine

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The standard therapy for growth hormone elevation in acromegaly has been conventional supervoltage irradiation. The data of human growth hormone levels in acromegalics who received radiotherapy has been evaluated in the ten-year level in 1976-77.

This project has been temporarily discontinued.

PHS-6040
Rev. 10-76

Project Number: ZO1 AM 47,014 - 11 DB

Project Description:

None

Publications:

1. Eastman, R. C., Gorden, P., and Roth, J.: Conventional supervoltage irradiation is an effective treatment for acromegaly. J. Clin. Endocrinol. Metab., 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,016 - 03 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Structure of the Insulin Receptor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. R. Kahn	Senior Surgeon	DB, NIAMDD
	J. T. Harmon	Staff Fellow/Guest Worker	DB, NIAMDD
	U. Lang	Guest Worker	DB, NIAMDD
	L. C. Harrison	Visiting Scientist	DB, NIAMDD
Other:	E. S. Kempner	Senior Investigator	LPB, NIAMDD
	W. Schlegel	Visiting Fellow	LNE, NIAMDD
	A. Chrambach	Senior Investigator	RRB, NICHHD

COOPERATING UNITS (if any)
Laboratory of Physical Biology, NIAMDD
Laboratory of Nutrition and Endocrinology, NIAMDD
Reproductive Research Branch, Natl. Inst. of Child Health and Human Development

LAB/BRANCH
Diabetes Branch

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The components of the insulin receptor have been examined in rat liver membranes and IM-9 lymphocytes using SDS polyacrylamide gel electrophoresis (PAGE) in conjunction with specific immunoprecipitation and radiation inactivation on SDS gels of surface labeled cells. The subunit structure of the insulin receptor of IM-9 membranes appears complex, containing 4 subunits; the largest of which has a mol. wt. of 80-100,000. This corresponds relatively closely to the size of the insulin binding site in rat liver membranes as determined by radiation inactivation. In addition, in the intact membrane there appears to be a high mol. wt. affinity regulatory protein.

9-3-6040
(Rev. 10-76)

Project Description:

Subunit Structure of the Insulin Receptor in IM-9 Cells

Solubilized insulin receptors behave as high molecular weight structures (about 1,000,000 daltons) on PAGE. When human lymphocytes (IM-9) were labeled with ^{125}I using lactoperoxidase and used to prepare a plasma fraction which was solubilized in Triton X-100, about 0.4% of the solubilized ^{125}I-proteins were immunoprecipitated by specific antireceptor IgG. There was a quantitative correlation between the precipitation of ^{125}I-proteins and ^{131}I-insulin prebound to the receptor. SDS-PAGE of immunoprecipitated ^{125}I-proteins revealed 4 components of 101,000, 81,000, 67,000 and 41,000 daltons. All peaks were eliminated by pretreatment of the membranes with trypsin. Preincubation of the solubilized receptor with insulin also caused a dose-and time-dependent suppression of all peaks, although the 41,000 peak was not completely eliminated. With more potent reducing conditions the 81,000 peak disappeared and the 41,000 peak increased. These data suggest a complex receptor structure consisting of 4 subunits with molecular weights of 41,000 to 101,000. The 81,000 dalton subunit appears to be a disulfide dimer of the 41,000 subunit. Blockade of immunoprecipitation of the 101,000, 81,000 and 67,000 dalton peaks by insulin suggests that all 3 participate in the insulin binding site.

Components of the Insulin Receptor in Rat Liver Membranes

Radiation inactivation is a technique which allows the determination of the molecular weight of a biologically functional molecule in the membrane. When membranes are exposed to high doses of high energy ionizing radiation, proteins are destroyed; the slope of the inactivation curve is a function of molecular volume or weight. Using this technique, we have determined the size of the insulin receptor in rat liver membranes. When rat liver membranes were irradiated complex results were observed. During the first 12 Mrad dose there was a 2-fold increase in specific insulin binding. Such an increase in the activity of a protein during irradiation has not been previously reported. In the case of the insulin receptor, the increased activity appeared to be due to an increase in receptor affinity as the result of inactivation of some high molecular weight molecule (350,000). This increase in binding was followed at higher radiation doses by a monoexponential decay in activity due to a decrease in receptor number (molecular weight of 90,000). Also, after exposure of membranes to high doses of radiation, there was a loss of negative-cooperative interactions as measured by dissociation experiments. Pre-treatment of the membranes with insulin caused the decay curve to be a monoexponential line with a slope corresponding to a molecular weight of 90,000. When receptor number was estimated by binding of ^{125}I-labeled antibodies to the insulin receptor, only a 90,000 dalton protein was observed. These data suggest that at least two membrane components are involved in the insulin receptor interaction: one with a molecular weight of 350,000 which appears to regulate the affinity of the insulin binding protein and is inactivated at low doses of radiation; and a second component with a molecular weight of 90,000 which binds the insulin. In the intact membranes the effect of the affinity-regulatory

Project Description Continued:

component is to lower the affinity of the insulin receptor for insulin. Insulin seems to induce a dissociation of the binding and affinity-regulatory units. Much of the insulin degrading activity appears distinct from the insulin receptor and has a higher molecular weight (1,000,000), although there is a component of 100,000 daltons.

Publications:

1. Lang, U., Kahn, C. R., and Chrambach, A.: Characterization of the insulin receptor from human lymphocytes by quantitative polyacrylamide gel electrophoresis. Endocrinol., 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,017 - 02 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Demonstration and Chracterization of Ouabain-like Activity in Amphibian Skin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. S. Flier	Surgeon	DB, NIAMDD
Other:	P. Nakada	Grant Supported Tech	DB, NIAMDD
	J. Daly	Research Chemist	LC, NIAMDD

COOPERATING UNITS (if any)

Laboratory of Chemistry, NIAMDD

LAB/BRANCH
Diabetes Branch

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX(c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been discontinued.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,018 - 02 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cellular Insulin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Rosenzweig	Clinical Associate	DB, NIAMDD
	J. Havrankova	Guest Worker	DB, NIAMDD
	M. A. Lesniak	Research Chemist	DB, NIAMDD
Other:	M. S. Brownstein	Medical Officer	LCS, NIMH
	J. Roth	Medical Director	DB, NIAMDD
	B. Samuels	Research Assistant	DB, NIAMDD

COOPERATING UNITS (if any)
Laboratory of Clinical Medicine, National Institute of Mental Health

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	0.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin has been found to be present in a wide variety of rat and human tissues in concentrations higher than plasma. The insulin present in extracts of rat liver was shown to be genuine insulin by its elution pattern on Sephadex chromatography, by serial dilution in the insulin radioimmunoassay, by its activity in the glucose oxidation bioassay and by neutralization of bioactivity with anti-insulin antibodies. Liver insulin (like brain insulin) did not change with fasting or with streptozotocin treatment in the rat, or in the ob/ob mouse, conditions associated with markedly altered insulin secretion and receptor content. Genuine insulin was also detected and characterized in IM-9 cultured human lymphocytes and in human fibroblasts in concentrations similar to tissue levels and as much as 50 to 100 times the insulin concentration in the media. Through the use of a monoclonal anti-beef insulin antibody, the insulin in the cells resembles human insulin more than it does beef insulin, the insulin in the media in which they were grown. The results suggest that a good part if not all of the insulin bound to the extrapancreatic tissues may be synthesized in the tissues themselves.

2[]-6040
(Rev. 10-76)

Project Description:

Recently, we have demonstrated the presence of insulin in the brain in concentrations multifold higher than plasma. It was shown to be genuine insulin by radioimmunoassay, gel filtration, radioreceptor assay and bioassay, and the brain levels of insulin are unchanged by the extremes of hyperinsulinemia and hypoinsulinemia.

In the present studies, we have shown that insulin is also present in signficant quantities in essentially all tissues of the rat. Immunoreactive insulin in the rat was measured in extracts of heart, kidney, skeletal muscle, spleen, lung, testis, brain, small intestine, liver and fat. The amount of immunoreactive insulin in the extracts represented tissue insulin concentrations from 2 to 10 times plasma levels. When values were corrected for losses of ^{125}I-insulin in the extraction procedure, tissue levels were as much as 5 to 100 times plasma levels.

Rat liver insulin was shown to be identical to genuine insulin by serial dilution in the RIA, Sephadex G-50, filtration, activity in the gluocose oxidation bioassay in isolated rat adipocytes, and by neutralization of the bioactivity with anti-insulin antibody. Rat liver insulin (like brain insulin) does not change with fasting or with streptozotocin treatment in the rat or in the ob/ob mouse, suggesting that liver insulin content is independent of the plasma insulin over the entire range of physiological extremes.

Insulin was found in a wide variety of human tissues as well, in concentrations higher than plasma. In order to examine cellular insulin in a more defined experimental situation, human cells in culture were studied. IM-9 lymphoblastoid cells were found to have insulin in concentrations averaging 20 ng/ml of cell volume, or as much as 100 times the concentration of insulin that could have been contributed by fetal calf serum in the media. Insulin was also found in similar concentrations in human fibroblasts. IM-9 human lymphocyte insulin was indistinguishable from genuine insulin by migration on G-50 Sephadex, by serial dilution in the RIA, by activity in the glucose oxidation bioassay and by neutralization of bioactivity with anti-insulin antibody. The cultured human cell insulin would not bind to a monoclonal anti-beef insulin antibody, suggesting that it resembles human insulin more than it does beef insulin; the insulin in the fetal calf serum in the media.

These results suggest that a good part, if not all of the insulin that we are finding in the extrapancreatic tissues may be synthesized by the tissues themselves. The physiological mechanisms and functions which govern cellular insulin and its synthesis are now under study.

Publications:

1. Havrankova, J., Schmechel, D., Roth, J., and Brownstein, M. S.: Identification of insulin in rat brain. Proc. Natl. Acad. Sci., USA 75: 5737-5741, 1978.

Publications Continued:

2. Havrankova, J., and Roth,J.: Concentrations of insulin and of insulin receptors in the brain are independent of peripheral insulin levels: Studies of obese and streptozotocin-treated rodents. J. Clin. Invest., in press.

Awards:

G. Burroughs Mider Award Lectureship awarded to J. Roth, September, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,019 - 02 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Morphological Studies of Ligand Binding to Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Gorden	Senior Surgeon	DB, NIAMDD
	J.-L. Carpentier	Chargé de Recherches	Univ of Geneva
	E. Van Obberghen	Visiting Associate	DB, NIAMDD
	M. A. Lesniak	Research Chemist	DB, NIAMDD
Other:	L. Orci	Prof of Medicine	Univ of Geneva
	P. Freychet	Prof of Medicine	Univ of Nice
	A. Le Cam	Chargé de Recherches	Univ of Nice
	S. Cohen	Prof of Biochemistry	Vanderbilt Univ

COOPERATING UNITS (if any)
Institute of Histology and Embryology, University of Geneva, Geneva, Switzerland; Department of Medicine, University of Nice, Nice, France; Department of Biochem., Vanderbilt University, Nashville, Tennessee

LAB/BRANCH
Diabetes Branch

SECTION
Clinical and Cellular Biology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

^{125}I-insulin binds to IM-9 cells (human) and hepatocytes (rat). By using quantitative EM autoradiography as a probe, it can be shown that labeled insulin is internalized by a process which appears to be time and temperature-dependent. Other labeled proteins (low density lipoprotein, epidermal growth factor, human growth hormone, glucagon, and anti-insulin receptor antibody) appear to behave in the same fashion i.e. initial binding followed by internalization.

PHS-6040
(Rev. 10-76)

Project Description:

This study was designed to visualize the interaction of a ligand with a cell from initial binding through steady-state binding. In as much as kinetic states of polypeptide hormones as well as many other ligands are done with ^{125}I-labeled material we chose EM autoradiography as our visual probe.

Our initial studies demonstrated that the labeled ligand bound to the plasma membrane at early time points of incubation and low temperatures but with increasing temperature and time there was progressive internalization of the ligand. We have now extended these studies to insulin in cultured human lymphocytes, and rodent hepatocytes. In addition, we have studied epidermal growth factor in human fibroblasts as well as low density lipoprotein in human fibroblasts. These studies all reveal a similar qualitative process that has initial binding followed by internalization. In all of these systems the internalized label material preferentially localizes in lysosomal structures. When the volume density of these lysosomal structures are studied quantitatively, it is found that they are increased in regions with autoradiographic grains. Thus, the binding is occurring to lysosomal stuctures which preferentially reside in the Golgi region of the cell.

It would appear that this is a general mechanism related to multiple different types of ligands including the above mentioned as well as anti-insulin receptor antibody, glucagon, and growth hormone and that the process may function to remove the ligand from the cell surface and cause its degradation. In addition, other studies indicate that this may also be a mechanism by which the cell surface receptor is lost.

Publications:

1. Orci, L., Perrelet, A., and Gorden, P.: Less-understood aspects of the morphology of insulin secretion and binding. In Greep, R. O. (Ed.): Recent Progress in Hormone Research, New York, Academic Press, Inc., 1978, Volume 34, pp. 96-121.

2. Gorden, P., Carpentier, J.-L., Cohen, S., and Orci, L.: Epidermal growth factor: Morphological demonstration of binding, internalization and lysosomal association in human fibroblasts. Proc. Natl. Acad. Sci. USA, 75: 5025-5019, 1978.

3. Carpentier, J.-L., Gorden, P., Freychet, P., Le Cam, A., and Orci, L.: Lysosomal association of internalized ^{125}I-insulin in isolated rat hepatocytes. Direct demonstration by quantitative electron microscopic autoradiography. J. Clin. Invest. 63: 1249-1261, 1979.

4. Carpentier, J.-L., Gorden, P., Barazzone, P., Freychet, P., Le Cam, A., and Orci, L.: Intercellular localization of ^{125}I-insulin in hepatocytes from intact rat liver. Proc. Natl. Acad. Sci., USA 76: 2803-2807, 1979.

5. Carpentier, J.-L., Gorden, P., Freychet, P., Le Cam, A., and Orci, L.: ^{125}I-insulin is preferentially internalized in regions of the hepatocytes rich in lysosomal and Golgi structures. Experientia, in press.

6. Carpentier, J.-L., Gorden, P., Goldstein, J. L., Anderson, R. G. W., Brown, M. S., and Orci, L.: Binding and internalization of ^{125}I-LDL in normal and mutant human fibroblasts: A quantitative autoradiographic study. Exp. Cell Res., in press.

7. Gorden, P., Carpentier, J.-L., Van Obberghen, E., Barazzone, P., Roth, J., and Orci, L.: Insulin-induced receptor loss in the cultured human lymphocyte: Quantitative morphological perturbations in the cell and plasma membrane. J. Cell Sci., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,020 - 02 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Insulin Antibodies, Insulin Allergy, and Insulin Resistance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. R. Kahn	Senior Surgeon	DB, NIAMDD
Other:	A. Rosenthal	Senior Investigator	LCI, NIAID
	D. Mann	Senior Investigator	I, NCI

COOPERATING UNITS (if any)
Laboratory of Clinical Investigation, Natl. Inst. of Allergy and Infectious Dis.
Immunology Branch, National Cancer Institute

LAB/BRANCH
Diabetes Branch

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with insulin allergy and insulin resistance were studied. Anti-insulin antibody titers, skin test reactivity to insulin, HLA type, and T-cell proliferation to insulin were measured. The immunologic determinants of insulin were identified and correlated without HLA type and clinical data.

PHS-6040
Rev. 10-76)

Project Number: Z01 AM 47,020 - 02 DB

Project Description:

Immunologic reactions, especially insulin allergy, are complications of insulin therapy. Recent studies in animals have suggested that the immune response to insulin is under genetic control and that within a species different inbred strains recognize different determinants of the molecule. In the present study, we have examined the specificity of T-cell antigen recognition in man by measuring ^{3}H-thymidine uptake in response to insulin or related antigens. One hundred and fifteen patients with insulin allergy or immunologic resistance were studied. Fifty-seven had positive and approximately equally proliferative responses to beef and pork insulins suggesting recognition of the common B-chain determinant (B-30). Five patients responded only to beef insulin, and in 12 patients the responses to beef insulin were much greater than to pork, suggesting recognition of the altered A8, 9, and 10 types also. Twenty-one patients responded to beef C-peptide, as judged by stronger reactions to the proinsulin of the species than the insulin. Interestingly, protamine was a stimulus for the T-cell in 51% of patients, and in 7 cases was the only antigen recognized, suggesting that in some cases protamine may potentiate insulin allergy by binding insulin and presenting it to the T-cell ("schlepper phenomenon"). In only 16 patients could no antigen be identified using this approach. These data suggest that immunologic reactions to insulin in man, like those in animals are controlled by immune response genes which determine the precise amino acid sequences recognized by the T-cell.

Publications:

1. Kahn, C. R., and Rosenthal, A. S.: Immunologic reactions to insulin: Insulin allergy, insulin resistance and the autoimmune insulin syndrome, Diabetes Care, 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 47,021 - 01 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Insulin Receptors and Action in Cultured Human Fibroblasts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. R. Kahn	Senior Surgeon	DB, NIAMDD
	J. Podskalny	Chemist	DB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin binding and action has been studied in cultured human fibroblasts. These cells have an insulin sensitive glycogen synthase. A receptor defect has been found in cells of a patient with the Type A syndrome of insulin resistance and acanthosis nigricans.

PHS-6040
(Rev. 10-76)

Project Number: Z01 AM 47,021 - 01 DB

Project Description:

Glycogen synthase (GS) is an hormonally modulated cellular enzyme whose regulation is well worked out in the rat adipocyte. Insulin is active in converting the enzyme from a G-6-P dependent, phosphorylated form (D) to a glucose independent, dephosphorylated form (I). We have chosen to examine glycogen synthase in an accessible human tissue, the cultured human fibroblast, in hopes to define defects in insulin action in cultured cells from patients with diabetes mellitus or insulin resistant states. Conditions under which the enzyme can be measured reproducibility in these cells have been worked out. Insulin routinely causes an increase in the percent independent glycogen synthase activity in the fibroblast after a 30 min. incubation with hormone. 24 hours of preincubation of the cells with a serum-free, glucose-free medium increases the insulin response. In contrast to some other cell types, glucose has little effect on the percent independent activity. Under optimal conditions insulin increases glycogen synthase acutely in human fibroblasts by two- to four-fold. Insulin sensitive GS activity in 3T3-L1 fatty fibroblasts grown in our laboratory and in WISH cells grown in collaboration with Fumio Shizumi and Abner Notkins. Further studies are planned to examine the regulation of the enzyme in cells cultured from diabetic subjects and to also use this as a routine bioactivity assay of insulin action in fibroblasts.

Cells from a patient with the Type A syndrome of insulin resistance and acanthosis nigricans have also been put into culture and examined for ^{125}I-insulin binding and bioactivity. Patient's cells, when compared to cells from control subjects, exhibit lower overall binding with an increase in the concentration of insulin required to give half-maximal displacement. It appears that the lower binding is due to a decrease in receptor affinity. Kinetic experiments reveal increased dissociation rate with little effect of cold insulin to accelerate dissociation, i.e. there is a loss of negative cooperativity. Despite this defect insulin stimulation of ^{3}H-thymidine incorporation into DNA is normal, and insulin stimulation of glycogen synthase still occurs.

Publications:

None

ANNUAL REPORT OF THE PEDIATRIC METABOLISM BRANCH
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

In the fiscal year 1978-1979 investigations on the generalized inherited disease, cystic fibrosis, have continued with special emphasis on the role of the autonomic nervous system, and polyamines as well as clinical studies.

I. POLYAMINE STUDIES

An abnormality in the distribution of polyamines has been recognized in cystic fibrosis. However, as the physiological role of these ubiquitous compounds is not well understood an effort is underway to further clarify their physiologic and pathologic role.

Biosynthesis of 2-Pyrrolidone from Putrescine

The diamine putrescine and the polyamines spermidine and spermine are ubiquitous in animal tissues. These compounds as well as their biosynthetic enzymes increase in the early phase of all growth processes and numerous hormones stimulate their synthesis in various target tissues. Based on the above observations these cations are presently viewed as important metabolites in growth and differentiation. The polyamine biosynthetic enzymes, as well as some of the mechanisms regulating their synthesis, have been characterized. In contrast, although several products of polyamine metabolism have been identified, far less is known about the enzymatic steps leading to their synthesis as well as the manner in which these pathways are regulated.

During initial studies undertaken to gain a better understanding of polyamine catabolism in sliced rat liver, a metabolic product derived from putrescine was observed to be secreted into incubation medium in relatively large quantities. Under similar assay conditions, but to a lesser extent, lung and spleen slices also secreted this metabolite in the presence of putrescine. On the other hand, none of the unknown was observed in medium after incubating putrescine with brain, kidney or muscle slices.

Preliminary steps taken to identify the unknown putrescine metabolite were unsuccessful. Unlike its cationic precursor it does not bind to cationic exchange resins, is not ninhydrin positive and runs with the solvent front in a variety of solvent systems including chloroform methanol. Subsequent experiments revealed the following properties of the putrescine metabolite: (a) Hydrolysis with 6N HCl yield γ-amino-butyric acid as determined with an amino acid analyzer. (b) It is volatile under several conditions (e.g. lyophilization or flash evaporation at 76°). (c) It has little affinity for either anionic or cationic exchange resins. (d) It is soluble in water, methanol and chloroform but not petroleum ether. (e) It absorbs to activated charcoal and can be eluted with an ethanolic ammonia solution. The above properties suggested the unknown might be 2-pyrrolidone, a compound not previously

known to be synthesized in any biological system.

When analyzed via gas chromatography the unknown metabolite had the same retention time as authentic 2-pyrrolidone. Identification of the [^{14}C] putrescine metabolite was made by recrystallizing authentic 2-pyrrolidone to which the [^{14}C] labelled unknown had been added. The specific activity (dpm/g 2-pyrrolidone) remained virtually constant after repeated recrystallizations. Formation of 2-pyrrolidone from putrescine by rat liver slices was shown to be dependent on time, tissue weight, and substrate concentration.

We are presently examining in greater detail under what conditions 2-pyrrolidone is formed from putrescine. In addition experiments have been initiated to purify and characterize the enzyme(s) involved in its synthesis.

It is of interest to note that hydrolysis of 2-pyrrolidone yields γ-aminobutyric acid an important compound in neurohumoral transmission. Relative to γ-aminobutyric acid and putrescine, 2-pyrrolidone is nonpolar and may pass through the blood-brain barrier. (Dr. David Lundgren)

II. THE AUTONOMIC NERVOUS SYSTEM IN CYSTIC FIBROSIS

We have examined the problem of autonomic nervous system activity in patients with CF at several levels.

Physiologic studies

Alpha adrenergic responsiveness was determined by measuring the concentration of topical phenylephrine required to cause pupillary dilation $\geq$0.5 mm. Patients with CF required significantly lower concentrations of phenylephrine than healthy nonallergic control subjects ($p<0.00001$) or patients with allergic asthma ($p<0.05$). The dose of phenylephrine required for pupillary dilation correlated significantly with clinical score (that is, the most severely ill patients were the most sensitive to topical phenylephrine), but no patient, even those with clinical scores over 90, required a dose of phenylephrine in the normal range. The atopic subjects with CF (3 of 11 subjects) did not account for this α-adrenergic hypersensitivity. Moreover obligate heterozygotes for CF (parents of patients with CF) had significantly ($p<0.0001$) greater sensitivity than normals, but significantly less ($p<0.01$) than patients with CF.

Cholinergic responsiveness was measured by determining the concentration of topical carbamylcholine required for pupillary constriction $\geq$1 mm. The patients with CF required a significantly lower dose of carbamylcholine than healthy nonallergic subjects ($p<0.0001$) or patients with allergic asthma ($p<0.01$). There was no significant correlation between cholinergic sensitivity and clinical score, and the atopic patients with CF did not account for increased cholinergic sensitivity. Obligate heterozygotes for CF were significantly ($p<0.05$) more sensitive than normal subjects, but less than patients with CF ($0.1>p>0.05$).

The response to β adrenergic stimulation was measured by determining the dose of intravenously infused isoproterenol required to increase the pulse pressure by ≥ 22 mm Hg. Patients with CF required a significantly higher dose than healthy nonallergic subjects ($p<0.005$), but they did not differ from patients with allergic asthma. There was no significant correlation with clinical score, and the atopic subjects did not account for the β-adrenergic resistance. (Dr. Pamela Davis, NIAMDD; Dr. Michael Kaliner, NIAID; Dr. James Shelhamer, NIAID)

Catecholamine measurements

Plasma norepinephrine levels were measured in the resting state and in response to standing and isometric exercise. In all of these conditions plasma norepinephrine was the same in patients with CF and age-matched control subjects. The subjects with CF did have significantly lower blood pressure than the control subjects, and lower serum Na and Cl and higher serum K; these changes may be related to the high salt content of CF sweat and be unrelated to the autonomic nervous system. (Dr. P.B. Davis, NIAMDD; Dr. C.R. Lake, NIMH)

Biochemical analysis of β-adrenergic responses in CF

Lymphocytes and granulocytes from patients with CF have markedly reduced cAMP response to β-adrenergic stimulation compared to cells from healthy controls or patients with bronchiectasis. There is no difference in the β-adrenergic response in patients with and without pancreatic insufficiency, and no correlation with degree of illness as gauged by NIH clinical score. Obligate heterozygotes for CF have a mean response which is intermediate between healthy controls and CF patients and differ significantly from both ($p<0.02$). For these reasons it seems that this phenomenon may be gene-related and not a secondary consequence of disease.

To further analyze this system we studied leukocytes from patients with CF age 15-35 yr, in good to excellent condition, who took only pancreatic enzyme supplements, vitamins, and dioctyl sodium sulfosuccinate. The number of β-adrenergic receptors, measured by specific binding of ^{3}H-dihydroalprenolol, is normal in both granulocytes and lymphocytes. The estimated K_D for alprenolol is normal. Adenylate cyclase activity in granulocyte homogenates and lymphocyte membrane preparations from patients with CF is normal in the basal state and when stimulated by guanyl-5'yl-imidodiphosphate (GMPPMP), GMPPNP + prostaglandin E_1, or fluoride; however, isoproterenol-stimulated adenylate cyclase activity is markedly reduced. From this data it seems that the β-adrenergic resistance to CF is not related to availability of substrate or cofactors in the whole cells, since it persists in broken cell preparations, nor is it related to number of β-adrenergic receptors, their binding properties, or to adenlyate cyclase itself. The coupling of the β receptor to adenylate cyclase is implicated.

Because crude lymphocyte membrane preparations from patients with CF, as well as whole cells, display the impaired β-adrenergic responsive-

ness, it is clear that the cause of this defect resides with the cells themselves once developed and survives extensive washing, homogenization, and centrifugation at 25,000 x g. However, serum from patients with CF may contain anomalous small peptides or abnormal concentrations of normal serum constituents which might influence the β-receptor-adenylate cyclase system. We have shown that the abnormal levels of polyamines in CF do not account for the abnormal responses of whole cells. Exposure to normal plasma for 45 min does not produce the defect in normal cells. It is thus probable that the defect is cell-based.

We and others have attempted to study the cAMP response to β-adrenergic stimulation in the cultured skin fibroblasts, a system remote from possible effects of disease, malnutrition, or drugs. We found that time after subculture, culture conditions, composition of the test medium, and method of harvesting cells all had significant influences on the cAMP response of the fibroblasts to isoproterenol or prostaglandin E_1. However, intrastrain variation was large compared to interstrain variation and no conclusions could be drawn regarding population differences. (Dr. Pamela Davis, NIAMDD: Dr. C.R. Lake, NIMH)

Summary

All these data, taken together, suggest that the phenotype and genotype of CF is marked by a constellation of autonomic nervous system abnormalities which include α-adrenergic and cholinergic hyperresponsiveness and β-adrenergic resistance, and that these abnormalities might 1) contribute to the pathophysiology of CF and 2) be symptomatic of a more general membrane dysfunction in CF.

III. CLINICAL INVESTIGATIONS

1. Adults with CF: Based on a study of 75 patients of ours between 18 and 48 years of age and an additional 232 cases of adults with CF from the literature we have characterized this syndrome in the older age group. It presents important differences with the clinical picture of cystic fibrosis in the pediatric age group and the relative incidence of certain complications leading in the older age group to medical and surgical emergencies has been defined. Clear recommendations as to the diagnostic and therapeutic approach in older patients are given, which should be of great use to internists and specialists in chronic pulmonary disease not sufficiently aware of this disorder even though it is the commonest cause of chronic obstructive pulmonary disease in the third decade of life. (Drs. P.A. di Sant'Agnese and P.B. Davis, NIAMDD)

2. Fatty-acid metabolism. Previously, we have demonstrated that the abnormal fatty-acid composition of plasma lipids observed in patients with CF is a secondary effect of the associated pancreatic insufficiency (Hubbard et al, Lancet 2:1302-1304, 1977). Further studies of the fatty acid analysis of both plasma lipids and erythrocyte membrane have substantiated our earlier findings to the extent that correct and accurate prediction of the exocrine pancreatic function of the patient could be identified by means of this analysis. There was no difference when

males and females were compared nor was there a correlation with the NIH clinical score. Older patients did demonstrate a more severe abnormality which only appeared to correlate with the duration of their pancreatic insufficiency. (Dr. V.S. Hubbard)

3. Cimetidine as an adjunct to pancreatic enzyme therapy. Since abnormal fatty-acid composition of lipids in CF patients has been thought to cause or potentiate alterations in all function and metabolism, therapeutic modalities are being sought to correct this abnormal finding. Cimetidine, an H_2 receptor antagonist which decreases gastric acidity at output, was given for 6 weeks to 8 CF patients with documented pancreatic insufficiency. Steatorrhea was reduced from a mean of 16% when taking enzymes to a mean of 9% when cimetidine and enzymes were taken. During the 6 weeks, minor changes in the fatty-acid composition of plasma lipids occurred which were suggestive of normalization, but a longer clinical trial is felt to be necessary before a definitive statement can be made. (Drs. V.S. Hubbard and L.A. Lester)

4. Evaluation of exocrine pancreatic function. The oral administration of a synthetic peptide, N-benzoyl-L-tyrosyl-p-amino-benzoic acid, which is a specific substrate for the proteolytic enzyme, chymotrypsin, has been evaluated for its predictive value of exocrine pancreatic function. If chymotrypsin is present within the intestinal fluid, digestion of this tripeptide occurs and p-amino-benzoic acid is absorbed, metabolized in the liver and excreted in the urine. Quantitation of aromatic amines in a timed urine collection following the administration of the test compound can be used to assess the presence of chymotrypsin. We have currently evaluated 22 CF patients with pancreatic insufficiency, 4 CF without clinical evidence of pancreatic dysfunction, 2 Shwachman-Diamond patients and 1 patient with unexplained malabsorption as well as normal volunteers. Results have been in agreement with other tests used to assess digestion and malabsorption such as 72 hour quantitative stool fat, serum carotene, results of fatty-acid analysis of plasma lipids and recovery of enzyme activity in duodenal fluid following duodenal intubation. These findings suggest that this test will be simple and useful for assessment of exocrine pancreatic function. Presently, improved methods of determination of the aromatic amines in the urine are being investigated which will make this test a simple office procedure available to all physicians. (Dr. V.S. Hubbard)

5. Echocardiographic assessment of cardiac performance in CF patients: Echocardiographic studies were done on 23 cystic fibrosis patients as part of a complete pulmonary evaluation that included chest x-ray, pulmonary function testing and EKG; of these, 11 were felt to have mild pulmonary disease, 8 moderate and 4 were severely affected and had overt signs of cor pulmonale. Preliminary results suggest that patients with clinically mild disease may start to develop right heart changes (increased right ventricular wall thickness and/or enlargement) at an earlier point in time than might have been predicted. Mild and moderately affected patients have normal left ventricular parameters and 1 of 4 patients with severe pulmonary disease was found to have significant left ventricular dysfunction, not clearly evident clinically. Serial

studies have been limited but in 1 patient improvement of right ventricular systolic time interval measurements (an indirect assessment of degree of pulmonary hypertension) was documented with significant clinical improvement. It is anticipated that further echocardiographic studies will provide useful information concerning the course of development of right heart changes in CF patients, and serial studies appear warranted to evaluate efficacy of various therapeutic interventions. Initiation of a study to evaluate the short term effects of prophylactic digoxin in selected patients is planned. (Dr. L. Lester, NIAMDD)

6. Additional clinical studies: Additional clinical studies have included an evaluation of *exocrine pancreatic function* in CF clearly showing that insulin and glucogon levels are affected only in patients with pancreatic function and reinforcing the impression that the so-called "diabetes" in cystics is secondary to "strangulation" of the islet cells of the pancreas by fibrosis. *Abdominal ultrasonography* was utilized to study the increased incidence of cholelithiasis in CF patients. A variety of *investigational anti-Pseudomonas antibiotics* was used including azlocillin and piperacillin to try to cope with the chronic respiratory infection with Pseudomonas aeruginosa which is a major problem in the treatment of patients with CF. Two cases of *human growth hormone deficiency* in conjunction with CF have been documented and treated in our clinic and are being reported. (Drs. P.A. di Sant'Agnese, V.S. Hubbard, P.B. Davis, T.L. Guidotti, L.A. Lester, NIAMDD)

Behavioral studies in CF have also been conducted and indicate that the unique set of life experiences of CF children has no apparent deleterious effect on their level of intellectual functioning, but in fact they contribute toward maximizing certain abilities. (Drs. H.A. Moss, and E.D. Nannis, NIMH and Dr. V.S. Hubbard, NIAMDD).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 49000-06 PMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biochemical and Physiological Studies of Polyamines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: David W. Lundgren, Ph.D. Senior Staff Fellow PMB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pediatric Metabolism Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The diamine putrescine and the polyamines spermidine and spermine are ubiquitous in animal tissues and are important compounds in growth and differentiation. Studies concerning the origin, fate, regulation and metabolic role of circulating polyamines are being continued. In addition, metabolism of [^{14}C] putrescine was studied in sliced rat liver. A new metabolite, 2-pyrrolidone, was found to be the major product of putrescine metabolism in incubation medium.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

Circulating polyamines are abnormal in individuals with cystic fibrosis as well as in blood from some cancer patients. The significance of this abnormality in CF is obscure, however, polyamines are the first well charachterized metabolite of low molecular weight demonstrated to be abnormal in CF. Studies of polyamine biochemistry and physiology are therefore being actively pursued. These studies should enhance our understanding of the role of polyamines in normal physiology.

Methods:

Biosynthesis of 2-Pyrrolidone from Putrescine

The diamine putrescine and the polyamines spermidine and spermine are ubiquitous in animal tissues. These compounds as well as their biosynthetic enzymes increase in the early phase of all growth processes and numerous hormones stimulate their synthesis in various target tissues. Based on the above observations these cations are presently viewed as important metabolites in growth and differentiation. The polyamine biosynthetic enzymes, as well as some of the mechanisms regulating their synthesis, have been characterized. In contrast, although several products of polyamine metabolism have been identified, far less is known about the enzymatic steps leading to their synthesis as well as the manner in which these pathways are regulated.

During initial studies undertaken to gain a better understanding of polyamine catabolism in sliced rat liver, a metabolic product derived from putrescine was observed to be secreted into incubation medium in relatively large quantities. Under similar assay conditions, but to a lesser extent, lung and spleen slices also secreted this metabolite in the presence of putrescine. On the other hand, none of the unknown was observed in medium after incubating putrescine with brain, kidney or muscle slices.

Preliminary steps taken to identify the unknown putrescine metabolite were unsuccessful. Unlike its cationic precursor it does not bind to cationic exchange resins, is not ninhydrin positive and runs with the solvent front in a variety of solvent systems including chloroform methanol. Subsequent experiments revealed the following properties of the putrescine metabolite: (a) Hydrolysis with 6N HCl yield γ-aminobutyric acid as determined with an amino acid analyzer. (b) It is volatile under several conditions (e.g. lyophilization or flash evaporation at 76°). (c) It has little affinity for either anionic or cationic exchange resins. (d) It is soluble in water, methanol and chloroform but not petroleum ether. (e) It absorbs to activated charcoal and can

be eluted with an ethanolic ammonia solution. The above properties suggested the unknown might be 2-pyrrolidone, a compound not previously known to be synthesized in any biological system.

When analyzed via gas chromatography the unknown metabolite had the same retention time as authentic 2-pyrrolidone. Identification of the [^{14}C] putrescine metabolite was made by recrystallizing authentic 2-pyrrolidone to which the [^{14}C] labelled unknown had been added. The specific activity (dpm/g 2-pyrrolidone) remained virtually constant after repeated recrystallizations. Formation of 2-pyrrolidone from putrescine by rat liver slices was shown to be dependent on time, tissue weight, and substrate concentration.

We are presently examining in greater detail under what conditions 2-pyrrolidone is formed from putrescine. In addition experiments have been initiated to purify and characterize the enzyme(s) involved in its synthesis.

It is of interest to note that hydrolysis of 2-pyrrolidone yields γ-aminobutyric acid an important compound in neurohumoral transmission. Relative to γ-aminobutyric acid and putrescine, 2-pyrrolidone is nonpolar and may pass through the blood-brain barrier.

Significance to Biomedical Research:

The polyamines have been shown to be abnormal in blood and urine of cystic fibrosis patients as well as in some cancer patients. However, the relationship between increased polyamine concentrations and various diseased states remains obscure. A better understanding of the physiology and biochemistry of polyamines may shed light on the native of cystic fibrosis and/or cancer.

Publications:

Lundgren, D.W. and Hankins, J.: Metabolism of putrescine to 2-pyrrolidone by rat liver slices. J. Biol. Chem.253: 7130-7133, 1978.

Lundgren, D.W. and Oka, T.: Alterations in polyamine levels in rat blood during pregnancy and lactation. Am. J. Physiol. 234: (5), E451-E456, 1978.

Sakai, T., Lundgren, D.W. and Oka, T.: Polyamine biosynthesis and DNA synthesis in cultured mammary gland explants from virgin mice. J. Cell Physiol. 95: 259-268, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 49001-06 PMB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Clinical Studies in Cystic Fibrosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P.A. di Sant'Agnese Chief PMB NIAMDD

OTHER: P.B. Davis, M.D., Ph.D. Surgeon PMB NIAMDD
T.L. Guidotti, M.D. Surgeon PMB NIAMDD
V.S. Hubbard, M.D., Ph.D. Senior Surgeon PMB NIAMDD
L.A. Lester, M.D. Senior Staff Fellow PMB NIAMDD

COOPERATING UNITS (if any)
Ronald Kahn, M.D. NIAMDD
Robert Wolf, D.D.S. NIDR
Howard Moss, Ph.D. NIMH

LAB/BRANCH
Pediatric Metabolism Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3	2

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cystic fibrosis (CF) is a generalized exocrinopathy in which the basic defect is still unknown. Studies have been pursued to determine the characteristics of the disease which are primary and related to a gene defect rather than secondary manifestations. The abnormal fatty acid pattern in patients with CF has been found to be a secondary effect of pancreatic insufficiency. Dysfunction of the endocrine system within the pancreas is also only seen in those patients with pancreatic insufficiency.

New diagnostic methods for evaluating the degree of pancreatic involvement have been investigated. The use of a specific substrate for chymotrysin which can be given orally and which yields a metabolite which is excreted in the urine has been found to be reliable. Ultrasonography also appears to have a predictive value of the degree of fibrosis present in the pancreas.

Echocardiography has been employed to demonstrate secondary cardiac involvement to be present even in patients with clinically mild disease.

PHS-6040
(Rev. 10-76)

Project Description:

Clarify the significance, incidence, diagnosis and therapy of various abnormalities and complications observed in CF patients of different age groups through the development of clinical studies, including the use of experimental diagnostic and antibiotic agents.

1. Studies on adults with cystic fibrosis. Cystic fibrosis is now the most common cause of chronic obstructive pulmonary disease (COPD) and of pancreatic insufficiency in the first three decades of life in the United States. We collected 75 patients with cystic fibrosis aged 18 to 47 years at the NIH and reviewed another 232 cases reported in the literature. All of these 307 patients had elevated sweat chloride and sodium levels, which proved excellent discriminants for cystic fibrosis even in patients in the older age group. COPD, present in 97 percent, was the major cause of morbidity and mortality, and differed from COPD of other etiologies. The progressive downhill course in these patients was punctuated by recurrent symptomatic exacerbations of chronic bacterial bronchitis caused by Pseudomonas aeruginosa and Staphyloccocus aureus, and terminated in pulmonary insufficiency, cor pulmonale and death. COPD was complicated by minor hemoptysis in 60 per cent, massive hemoptysis in 7 per cent, and pneumothorax in 16 per cent, problems rare in children. Sinusitis was present in all those examined roentgenographically, and 48 per cent had nasal polyposis. Pancreatic insufficiency was present in 95 per cent of the patients, but in contrast to younger patients it was seldom symptomatic although steatorrhea and azotorrhea were still massive. Intussusception and meconium ileus equivalent (fecal accumulation) are frequent in adults (21 per cent) but rare in children, and they require immediate diagnostic and therapeutic intervention with enemas of diatrizoate sodium. Glycosuria, biliary cirrhosis, cholelithiasis and aspermia were among other complications. Height and weight were usually within the lower limits of normal, but 17 per cent of the men were above 180 cm in height and 7 per cent were overweight.

2. Fatty-acid metabolism. Previously, we have demonstrated that the abnormal fatty-acid composition of plasma lipids observed in patients with CF is a secondary effect of the associated pancreatic insufficiency (Hubbard et al, Lancet 2:1302-1304, 1977). Further studies of the fatty acid analysis of both plasma lipids and erythrocyte membrane have substantiated our earlier findings to the extent that correct and accurate prediction of the exocrine pancreatic function of the patient could be identified by means of this analysis. There was no difference when males and females were compared nor was there a correlation with the NIH clinical score. Older patients did demonstrate a more severe abnormality which only appeared to correlate with the duration of their pancreatic insufficiency.

3. Cimetidine as an adjunct to pancreatic enzyme therapy. Since abnormal fatty-acid composition of lipids in CF patients has been thought to cause or potentiate alterations in all function and metabolism, therapeutic modalities are being sought to correct this abnormal finding. Cimetidine, an H_2 receptor antagonist which decreases gastric acidity at output, was given for 6 weeks to 8 CF patients with documented pancreatic insufficiency. Steatorrhea was reduced from a mean of 16% when taking enzymes to a mean of 9% when cimetidine and enzymes were taken. During the 6 weeks, minor changes in the fatty-acid composition of plasma lipids occurred which were suggestive of normalization, but a longer clinical trial is felt to be necessary before a definitive statement can be made.

4. Evaluation of exocrine pancreatic function. The oral administration of a synthetic peptide, N-benzoyl-L-tyrosyl-ρ-amino-benzoic acid, which is a specific substrate for the proteolytic enzyme, chymotrypsin, has been evaluated for its predictive value of exocrine pancreatic function. If chymotrypsin is present within the intestinal fluid, digestion of this tripeptide occurs and ρ-amino-benzoic acid is absorbed, metabolized in the liver and excreted in the urine. Quantitation of aromatic amines in a timed urine collection following the administration of the test compound can be used to assess the presence of chymotrypsin. We have currently evaluated 22 CF patients with pancreatic insufficiency, 4 CF without clinical evidence of pancreatic dysfunction, 2 Shwachman-Diamond patients and 1 patient with unexplained malabsorption as well as normal volunteers. Results have been in agreement with other tests used to assess digestion and malabsorption such as 72 hour quantitative stool fat, serum carotene, results of fatty-acid analysis of plasma lipids and recovery of enzyme activity in duodenal fluid following duodenal intubation. These findings suggest that this test will be simple and useful for assessment of exocrine pancreatic function. Presently, improved methods of determination of the aromatic amines in the urine are being investigated which will make this test a simple office procedure available to all physicians. Amylase isozymes are being determined in the serum of the above patients as an alternative method (or additional method) of assessing exocrine pancreatic function. By comparing the different methods of assessment, we hope to determine which tests are most reliable, especially in consideration of the time, cost, and discomfort to the patient.

5. Endocrine pancreatic function in CF. It was postulated by previous studies in our branch in conjunction with the CE, NIAMDD (N. Engl. J. Med. 281: 451, 1969) that the glucose intolerance in many of the CF patients was due to the increasing fibrosis of the pancreas leading to "strangulation" of the islets and α and β cell dysfunction. We have attempted to further define the endocrine function of the pancreas in relationship to the exocrine function of the pancreas within our CF patients. Four hour oral glucose tolerance tests were performed on 23 CF patients and 8 normal volunteers with glucose and insulin determinations being made. Additionally,

12 of the CF patients including 5 patients with normal exocrine pancreatic function underwent infusions with L-arginine as a stimulation test for glucagon. Only patients with evidence of exocrine pancreatic dysfunction demonstrated depressed levels of insulin and glucagon following these stimulation tests. However, some patients (50%) with pancreatic insufficiency and all patients without pancreatic insufficiency could not be distinguished from the normal volunteers. Additionally, follow-up of 6 surviving patients from the 1969 study have demonstrated no progression of glucose intolerance or insulinemia.

6. Abdominal ultrasonography. Many CF patients experience episodes of abdominal pain of unknown etiology. Since recent reports have shown a 12% incidence of cholelithiasis and a 40% incidence of abnormal oral cholecystograms, other means of evaluating the gall bladder is of clinical importance. Ultrasonograms are 2-dimensional laminographic images which do not depend on the function of the organ being examined and therefore are useful in patients with non-visualization of the gallbladder with oral cholecystograms. Approximately 20 patients have been examined with adequate visualization being obtained in all but 4 patients. Two of these 4 patients have subsequently expired and at necropsy the gallbladder was noted to be less than 1 x 2 cm and almost atretic.

Additionally, density characteristics calculated from ultrasourd of the pancreas have recently been found to correlate with the degree of pancreatic dysfunction with the more dense pancreas being observed in those with pancreatic insufficiency. Though this may not be a surprising finding in patients with CF, it does help to correlate observations made through advanced techniques of ultrasonography and the pathophysiology of disease.

7. Echocardiographic assessment of cardiac performance in CF patients: Echocardiographic studies were done on 23 cystic fibrosis patients as part of a complete pulmonary evaluation that included chest x-ray, pulmonary function testing and EKG; of these, 11 were felt to have mild pulmonary disease, 8 moderate and 4 were severely affected and had overt signs of cor pulmonale. Preliminary results suggest that patients with clinically mild disease may start to develop right heart changes (increased right ventricular wall thickness and/or enlargement) at an earlier point in time than might have been predicted. Mild and moderately affected patients have normal left ventricular parameters and 1 of 4 patients with severe pulmonary disease was found to have significant left ventricular dysfunction, not clearly evident clinically. Serial studies have been limited but in 1 patient improvement of right ventricular systolic time interval measurements (an indirect assessment of degree of pulmonary hypertension) was documented with significant clinical improvement. It is anticipated that further echocardiographic studies will provide useful information concerning the course of development of right heart changes in CF patients, and serial studies appear warranted to evaluate efficacy of various therapeutic interventions. Initiation of a study to

evaluate the short term effects of prophylactic digoxin in selected patients is planned.

8. Investigational anti-Pseudomonas antibiotics: Chronic respiratory infection with Pseudomonas aeruginosa is a major problem in the treatment of patients with CF. A strain of Pseudomonas resistant to the commonly available and used antibiotics are becoming more frequent. Azlocillin and piperacillin are two new penicillin derivatives which have shown excellent in vitro anti-Pseudomonas activities and are being evaluated in CF patients with serious pulmonary exacerbation by chronic Pseudomonas infection.

9. Children afflicted with life-threatening illnesses are subject to severe psycho-social stress which in turn could impair various aspects of their daily functioning. For example, sustained anxiety, constricted life space, withdrawal from a future perspective, and parental restrictiveness are all possible psychological consequences of a chronic, life-threatening illness which could interfere with optimal intellectual functioning.

As part of a larger study which investigated whether serious illness among children has resulted in any specific or general decrement of intellectual skills, a group of patients with cystic fibrosis (CF) and their healthy siblings were studied. None of the CF patients was being treated for exacerbation of symptoms of their illness at the time of the testing. Twenty-two patients ranging in age from 4 to 22 years and 20 of their healthy siblings ranging in age from 6 to 24 years old were administered the Wechsler Intelligence Scales. The Wechsler scales provide measures of both general and specific abilities and thus allow for assessing and comparing different levels of functioning. Since the healthy siblings had comparable environmental and genetic backgrounds to those of the CF patients they provided suitable control cases.

Analyses consisted of studying within group variations in abilities of the cystic fibrosis cases and comparisons of the scores of these children with those of their healthy sibs. The mean full scale IQ of the CF children was 118 whereas that of their sibs was 113. There was no significance within group variation among the CF patients. The children with CF did function at a significantly higher level than their sibs on the performance half of the Wechsler Intelligence Scales. (Performance IQ of 118 vs. 109, $t = 2.27$, $p < .05$, two-tailed) as well as on the specific subscale dealing with awareness of the details and organization of social situations.

These data indicate that the unique set of life experiences of CF children has no apparent deleterious effect on their level of intellectual functioning, but in fact, may contribute toward maximizing certain abilities.

10. Case studies: (a) Two patients with CF and human growth hormone deficiency have been documented and treated in our clinic. It is felt that this is a chance coexistence of the two disorders. However it is

important to recognize that most patients with CF do not fall below the 3rd percentile on the growth charts and if they are below the 1st percentile other etiologies of their short stature should be sought.

b. The possibility that timed-release aspirin may lead to an increased risk of gastrointestinal hemorrhage within the small intestine in patients with pancreatic insufficiency was postulated following the occurence of a massive GI bleed in a CF patient who was receiving treatment for disabling pulmonary osteoarthropathy. Altered intra-luminal environment in the lower duodenum and jejunum in those patients with decreased bicarbonate secretion makes the mucosa of the small intestine more susceptible to the ulcerogenic action of the aspirin.

c. A patient with a sibling with autopsy-documented CF, pulmonary disease typical of cystic fibrosis (upper lobe and right sided predominance of bronchiectatic lesions infected with mucoid Pseudomonas aeruginosa), sinusitis, nasal polyposis, and abnormal bicarbonate response to secretin but normal pancreatic enzyme release, and a sweat chloride of 20-44 mEq/l and sodium, 36-55 mEq/l was referred to us. Stimulated by this patient, we reviewed the results of sweat electrolyte determinations in patients with cystic fibrosis whose pancreatic function had been definitively evaluated. We found that those with pancreatic insufficiency had significantly ($p<0.0005$) higher sweat Na and Cl than those without pancreatic insufficiency. This observation gives rise to several testable hypothesis regarding the relationship of malnutrition even of minor degree to sweat electrolytes.

d. Two kinships with a previously unrecognized syndrome characterized by congenital bronchiectasis, normal sweat tests, absence of sinusitis, normal situs, normal ciliary ultrastructure including dynein arms and radial spokes, decreased sperm motility in males, normal immunological function and normal α_1-antitrypsin have been identified. One kinship has 3(2M, 1F) out of 7 affected sibs and the other 2(1M, 1F) out of 3 affected sibs. Further work-up of this syndrome is being pursued.

Significance to Biomedical Research:

Clarifying the basic hereditary defect in CF, the most frequent semi-lethal genetic disease in this country.

Defining and improving the diagnostic and therapeutic approach to the many manifestations and/or complications of patients with CF of various ages may vary dependent on their age, degree of pancreatic involvement, and development of secondary complications.

Publications

di Sant'Agnese, P.A. and Davis, P.B.: Cystic fibrosis in adults - 75 cases and a review of 232 cases in the literature. Am. J. Med. 66: 121-132, 1979.

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Hubbard, V.S., Davis, P.B., Schwartz, R.H., and di Sant'Agnese, P.A.: Isolated growth hormone deficiency and cystic fibrosis. Am. J. Dis. Child. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM-49003-04 PMB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (30 characters or less)
Cyclic Nucleotides and the Autonomic Nervous System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Pamela B. Davis, M.D., Ph.D. Clinical Associate PMB NIAMDD

COOPERATING UNITS (if any)
James H. Shelhamer, M.D. LCI NIAID
Michael Kaliner, M.D. LCI NIAID
Charles R. Lake, M.D. LCS NIMH

LAB/BRANCH
Pediatric Metabolism Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

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CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cystic fibrosis (CF) is a disorder primarily involving the exocrine glands, and we are studying cyclic nucleotides, which may regulate secretion in CF. Present topics are: (1) the concentration and distribution of cyclic AMP and cyclic GMP in normal and CF body fluids, (2) the response of cyclic AMP to β-receptor stimulation in normal and CF lymphocytes and fibroblasts, (3) the response of cyclic GMP to stimulation of the cholinergic receptor in several systems, (4) levels and response of physiologic stimuli of circulating norepinephrine, epinephrine, and dopaminehydroxylase, measures of sympathetic nervous system activity in CF, and (5) measurement of number and binding affinity of β-receptors in CF tissues.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The role of the cyclic nucleotides in exocrine secretory processes is still being determined, but it is suspected that they mediate the influence of the autonomic nervous system on the glands and may be essential determinants of secretion. Since cystic fibrosis is a disorder involving predominantly the exocrine glands, we are studying the physiology and distribution of cyclic nucleotides in CF, and their response in certain tissues to α- or β-adrenergic or cholinergic stimuli.

Physiologic studies

Alpha adrenergic responsiveness was determined by measuring the concentration of topical phenylephrine required to cause pupillary dilation $\geq$0.5 mm. Patients with CF required significantly lower concentrations of phenylephrine than healthy nonallergic control subjects ($p<0.00001$) or patients with allergic asthma ($p<0.05$). The dose of phenylephrine required for pupillary dilation correlated significantly with clinical score (that is, the most severely ill patients were the most sensitive to topical phenylephrine), but no patient, even those with clinical scores over 90, required a dose of phenylephrine in the normal range. The atopic subjects with CF (3 of 11 subjects) did not account for this α-adrenergic hypersensitivity. Moreover obligate heterozygotes for CF (parents of patients with CF) had significantly ($p<0.0001$) greater sensitivity than normals, but significantly less ($p<0.01$) than patients with CF.

Cholinergic responsiveness was measured by determining the concentration of topical carbamylcholine required for pupillary constriction $\geq$ 1 mm. The patients with CF required a significantly lower dose of carbamylcholine than healthy nonallergic subjects ($p<0.0001$) or patients with allergic asthma ($p<0.01$). There was no significant correlation between cholinergic sensitivity and clinical score, and the atopic patients with CF did not account for increased cholinergic sensitivity. Obligate heterozygotes for CF were significantly ($p<0.05$) more sensitive than normal subjects, but less than patients with CF ($0.1>p>0.05$).

The response to β adrenergic stimulation was measured by determining the dose of intravenously infused isoproterenol required to increase the pulse pressure by $\geq$ 22 mm Hg. Patients with CF required a significantly higher dose than healthy nonallergic subjects ($p<0.005$), but they did not differ from patients with allergic asthma. There was no significant correlation with clinical score, and the atopic subjects did not account for the β-adrenergic resistance.

Catecholamine measurements

Plasma norepinephrine levels were measured in the resting state and in response to standing and isometric exercise. In all of these conditions plasma norepinephrine was the same in patients with CF and age-matched control subjects. The subjects with CF did have significantly lower blood pressure than the control subjects, and lower serum Na and Cl and higher serum K; these changes may be related to the high salt content of CF sweat and be unrelated to the autonomic nervous system.

Biochemical analysis of β-adrenergic responses in CF

Lymphocytes and granulocytes from patients with CF have markedly reduced cAMP response to β-adrenergic stimulation compared to cells from healthy controls or patients with bronchiectasis. There is no difference in the β-adrenergic response in patients with and without pancreatic insufficiency, and no correlation with degree of illness as gauged by NIH clinical score. Obligate heterozygotes for CF have a mean response which is intermediate between healthy controls and CF patients and differ significantly from both ($p<0.02$). For these reasons it seems that this phenomenon may be gene-related and not a secondary consequence of disease.

To further analyze this system we studied leukocytes from patients with CF age 15-35 yr, in good to excellent condition, who took only pancreatic enzyme supplements, vitamins, and dioctyl sodium sulfosuccinate. The number of β-adrenergic receptors, measured by specific binding of [^{3}H]-dihydroalprenolol, is normal in both granulocytes and lymphocytes. The estimated K_D for alprenolol is normal. Adenylate cyclase activity in granulocyte homogenates and lymphocyte membrane preparations from patients with CF is normal in the basal state and when stimulated by guanyl-5'yl-imidodiphosphate (GMPPMP), GMPPNP + prostaglandin E_1, or fluoride; however, isoproterenol-stimulated adenylate cyclase activity is markedly reduced. From this data it seems that the β-adrenergic resistance to CF is not related to availability of substrate or cofactors in the whole cells, since it persists in broken cell preparations, nor is it related to number of β-adrenergic receptors, their binding properties, or to adenlyate cyclase itself. The coupling of the β receptor to adenylate cyclase is implicated.

Because crude lymphocyte membrane preparations from patients with CF, as well as whole cells, display the impaired β-adrenergic responsiveness, it is clear that the cause of this defect resides with the cells themselves once developed and survives extensive washing, homogenization, and centrifugation at 25,000 x g. However, serum from patients with CF may contain anomalous small peptides or abnormal concentrations of normal serum constituents which might influence the β-receptor-adenylate cyclase system. We have shown that the abnormal levels of polyamines in

CF do not account for the abnormal responses of whole cells. Exposure to normal plasma for 45 min does not produce the defect in normal cells. It is thus probable that the defect is cell-based.

We and others have attempted to study the cAMP response to β-adrenergic stimulation in the cultured skin fibroblasts, a system remote from possible effects of disease, malnutrition, or drugs. We found that time after subculture, culture conditions, composition of the test medium, and method of harvesting cells all had significant influences on the cAMP response of the fibroblasts to isoproterenol or prostaglandin E_1. However, intrastrain variation was large compared to interstrain variation and no conclusions could be drawn regarding population differences.

Summary

All these data, taken together, suggest that the phenotype and genotype of CF is marked by a constellation of autonomic nervous system abnormalities which include α-adrenergic and cholinergic hyperresponsiveness and β-adrenergic resistance, and that these abnormalities might 1) contribute to the pathophysiology of CF and 2) be symptomatic of a more general membrane dysfunction in CF.

Publications:

Davis, P.B., Braunstein, M. and Jay, C.: Decreased adenosine 3':5'-cyclic monophosphate response to isoproterenol in cystic fibrosis leukocytes. Pediat. Res. 12: 703-707, 1978.

Davis, P.B.: Urinary cyclic nucleotides in adult male cystic fibrosis patients. Clin. Chim. Acta 87: 285-288, 1978.

Lake, C.R., Davis, P.B., Ziegler, M. and Kopin, I.: Electrolytes and nonepinephrine levels in blood of patients with cystic fibrosis. Clin. Chim. Acta 92: 141-146, 1979.

ANNUAL REPORT SUMMARY

CLINICAL HEMATOLOGY BRANCH

I. Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

A. Relative Sensitivity of Platelets and Coronary Vasculature to Platelet-inhibitory Drugs of Clinical Importance

A number of drugs known to inhibit platelet function, e.g., sulfinpyrazone, ibuprofen, aspirin, and indomethacin may have value in treatment of coronary artery disease. These drugs not only block synthesis of prostaglandins that appear to be involved in platelet aggregation, but also block synthesis of prostaglandins in endothelium that promote vasodilation. In theory, optimal doses for coronary disease would inhibit prostaglandins involved in deleterious platelet aggregation, but not interfer with synthesis of possibly beneficial vasodilator prostaglandins. To determine whether it was possible to effect such optimal doses in vitro, we measured inhibition of vasodilation induced by arachidonic acid and in vitro platelet aggregation induced by ADP in response to various doses of platelet inhibitory drugs. Coronary circulatory studies were done in dogs, and platelet function studies were done on dog platelets taken at the same time circulatory measurements were made. All of the above mentioned drugs inhibited platelets at doses that produced comparable inhibition of coronary vasodilation; there appears to be no "optimal" dose that inhibits platelet aggregation without producing inhibition of synthesis of vasodilator prostaglandins. We are currently screening other platelet inhibitory drugs for their possible properties of specifically or more sensitively interfering with platelet function.

B. Relationships Between Platelet Function and Prostaglandin Metabolism

Arachidonic-acid-oxygenated products synthesized by human platelets have potent effects on platelet function suggesting that these compounds play a key role in platelet aggregation. The enzyme, cyclooxygenase, is markedly inhibited by the pharmacologic agent used clinically to depress platelet function as therapy for thromboembolic disorders. In evaluating the platelet physiologic and metabolic response to these various inhibitors, it has become important to measure the effects on prostaglandin and thromboxane synthesis. Assays for these compounds currently involved radioimmuno techniques or thin layer chromatography, but in recent years it has been realized that one of the metabolic products of oxygenated arachidonic acid compounds is malondialdehyde (MDA) which can be measured by a simple colormetric assay. We have explored the use of MDA measurement as an overall indication of platelet prostaglandin synthesis activity in response to a variety of physiologic

stimuli in the presence and absence of clinically useful platelet inhibitors. We found that the MDA assay permits unusually sensitive measurement of physiologic platelet stimulation and provides a sensitive indication of platelet responses to inhibitors listed in Section A above. We are currently exploiting this assay in the analysis of normal, inhibited, and abnormal platelet function in patients.

C. Isolation and Characterization of the Coagulant Moiety (f VIII) of the von Willebrand Factor - Factor VIII Complex

Antihemophilic globulin isolated in its purist form is a large molecular weight protein that has two functional activities: 1) promotes aggregation of platelets in the presence of von Willebrand factor (vWf), and 2) promotes coagulation of blood through the classical coagulation pathway (f VIII). Both the aggregating and coagulant actions of this protein have been considered the attributes of a single molecule with a monomer molecular weight of approximately 250,000 and a multimer of 2-5 million in plasma. Utilizing a new method of purification that we developed (last year's report) involving filtration of plasma through controlled pore glass, we found that the coagulant part of antihemophilic globulin could be separated from vWf at high ionic strength. Under these conditions, vWf appears in a fraction of molecular weight 2-5 million; whereas f VIII appears in a fraction of molecular weight approximately 250,000. When the isolated f VIII is rechromatographed at physiologic ionic strength, it does not tend to self-aggregate into multimers. By immunizing rabbits with isolated f VIII and utilizing antibodies that arise clinically in patients with idiopathic acquired hemophilia, we found that f VIII had immunologic characteristics distinct from vWf. These findings substantiate for the first time that antihemophilic globulin consists of two separate molecules. Various preparations of AHG used clinically in the treatment of either classical hemophilia or von Willebrand's factor contains different proportions of vWf and f VIII depending on the method of purification. Some of the fractions with highest purity contain relatively little of the vWf moiety and are, therefore, relatively ineffective in treating von Willebrand's disease. Work is progressing to further characterize the molecules that are deficient in plasma of patients with the different forms of hemophilia.

D. Methods for Preparing Isolated Intact Secretory Granules of Platelets

Platelet function involves secretion of biogenetic amines, calcium, and nucleotides from cytoplasmic granules in response to a variety of physiologic stimuli. Platelets are essentially a prototype of all secretory tissues and have been studied extensively with respect to the response of the intact cell to stimuli. In understanding the process of secretion, it has become important to determine whether stimuli that effect the intact cell are capable of affecting isolated platelet granules. Granules of other cells, for

example the adrenal medulla, have been isolated with relative ease compared to isolation of the whole cell; whereas granules of platelets have been difficult to obtain intact despite the ease of isolating the cells. This is apparently owing to the fact that platelet granular material acts in an autocatalytic manner to release contents of other platelet granules, and no method is available as yet to prevent granule disruption during isolation. We have explored various techniques to disrupt platelet plasma membranes, keeping granule membrane intact by systematically applying ultrasonication, nitrogen cavitation, and high pressure shearing techniques along with the use of enzymes or detergents to weaken plasma membranes with a variety of specific inhibitors to prevent release of granular material. So far the best preparations contain yields of only approximately 20% of the granules. We will continue to explore new techniques to improve yields in order to isolate sufficient granules to permit comparing their responses to those of intact cells.

E. Further Observations on Platelet Function in Homocystinuria

With J. Schulman of NICHD we have been following patients with homocystinuria due to cystathionine synthase deficiency because of their susceptability to thromboembolic disorders. Although some reports in the literature suggest that the underlying abnormality predisposing to thromboembolism is a tendency for platelets of these patients to be abnormally adherent to endothelium to the extent that platelet survival is shortened, we have not found this to be the case in six patients studied at NIH. Patients with homocystinuria have been treated with platelet inhibitors, dipyridamole and aspirin, as prophylaxis; but we have concluded from our studies that therapy directed at reducing clotting factors rather than platelet function might be more effective. Last year we reported a patient who developed thrombophlebitis and pulmonary embolisim while receiving antiplatelet therapy, and this year another of our patients receiving this conventional form of treatment developed the same problem. These are the first two reported cases of thromboembolic phenomena in homocystinuric patients receiving antiplatelet therapy. Our experience indicates that such patients should receive long-term therapy directed at reducing clotting factors (coumadin or heparin), rather than aspirin and dipyridamole.

II. Study of Immunology of Blood Cell Deficiencies

A. Characteristics of Abnormal Platelet Receptors for Antigen-Antibody Complexes and Isoantibodies

It has been known for several years that patients with certain inherited functional platelet disorders lack or have marked deficiencies of several glycoproteins identifable by polyacrylamide gel electrophoresis of their platelet membranes. For example, patients with thrombasthenia, lack glycoproteins which have been labeled IIba and III;, platelets of these patients do not aggregate in

reponse to all of the known platelet stimuli such as thrombin, collagen, epinephrin or antigen antibody reactions. Patients with the Bernard-Soulier syndrome lack glycoproteins GBId and GPIs, and their platelets do not aggregate with ristocetin in the presence of von Willebrand's factor. By chance it was also found that patients with thrombasthenia and Bernard-Soulier syndrome have aberrent reactions with certain antiplatelet antibodies. Patients with thrombasthenia that are geneotypically homozygous for the Pl^{A1} platelet antigen have been found to bind less than 10% of the normal amount of anti-Pl^{A1} and to show no complement fixation with this antibody. By equilibrium studies and elution analyses, it was found that the receptor for anti-Pl^{A1} on thrombasthenic platelets had much lower avidity than normal platelets, accounting for decreased absorption and failure to fix complement with this antibody. Although all human platelets tested so far (greater than 2,000 individuals) have reacted in a quantatively similar manner with antibodies directed against quinine and quinidine, it was found that patients with Bernard-Soulier syndrome fixed little or no complement with these drugs antibody, whereas absorption and elution studies showed that platelets from these patients absorbed at least 50% of the normal amount of the antibody. We are pursuing the observations in attempts to determine the significance of the lack of specific membranes glycoproteins in affecting the ability of platelets to react with iso- or drug antibodies either primarily through decreased absorption or secondarily through interference with complement fixation. This is the first time that a clearly identifiable inherited cellular membrane defect has been implicated in aberrent immuno-reactions involving isoantibodies and drug antibodies. It is hopeful that further study may lead to understanding the functional attributes of certain isoantigenic structures or receptors for drug-antibody complexes.

B. Therapy of Isoimmune Clinical Problems Involving Platelets

We have continued our studies on therapy of isoimmune thrombocytopenic states caused by maternal-fetal transfer of antiplatelet antibodies and by isoimmune antibodies arising in the syndrome of post-transfusion purpura which we described for the first time a number of years ago. Most of the cases of neonatal and post-transfusion purpura are caused by an antibody to the Pl^{A1} platelet antigen elicited either by transfer of antigenic platelets from the fetus to a Pl^{A1} negative mother or by antigenic transfused platelets in a Pl^{A1} negative recipient. The main approaches to treatment of the fulminant thrombocytopenic states have been exchange transfusion to rid patients of antibodies or antigen-antibody complexes, replacement with Pl^{A1} negative platelets, or infusion of Pl^{A1} postive platelets to absorb circulating antibody. In 14 cases of neonatal purpura in the past year our suggestion to use Pl^{A1} positive platelets as an antibody absorbent has been uniformly successful, whereas transfusion of maternal Pl^{A1} negative platelets has generally led to prolongation of the syndrome by transfer of small but significant amounts of antibody along with

maternal platelets. It is almost impossible to identify Pl^{A1} negative donors in the general population because less than 2% of individuals have this type of platelet. In post-transfusion purpura we have found that exchange transfusion utilizing Pl^{A1} positive donors has been the most successful form of treatment in two additional cases of this disorder, whereas one patient treated elsewhere with platelets from Pl^{A1} negative donors failed to respond and succumbed to purpura. These studies done in collaboration with referring physicians have been most helpful in establishing effective forms of therapy for these disorders.

III. Mechanisms of Hormone and Transmitter Secretion

A. Mechanisms of Calcium Action in Exocytosis

We have proposed that synexin, an adrenal medullary protein that causes Ca^{2+}-dependent aggregation of isolated chromaffin granules, might be the intracellular receptor for Ca^{2+} in the process of exocytosis. We now report that Ca^{2+} interacts directly with isolated synexin, inducing rapid self-association. Mg^{2+} or Sr^{2+} cannot substitute for Ca^{2+}, and Ba^{2+} is only weakly effective at stimulating self-association. We have analyzed 90° light scattering data to determine a titration curve for the activation of synexin by Ca^{2+} in the self-association reaction. The curve has a Hill coefficient of 2.3 and is half-maximal at 200 μM Ca^{2+}, which correlates exactly with the Ca^{2+} titration curve for the aggregation of chromaffin granules by synexin. Electron microscopy of negatively stained samples reveals that synexin monomers associate to form 50 A by 150 A rods which in turn associate side to side and end to end to form bundles of parallel rods. We suggest that synexin exists as a soluble monomer in the cytoplasm of the resting chromaffin cell and that when the cell is stimulated to secrete, the increase in the intracellular concentration of Ca^{2+} causes synexin to form rods, similar to those formed in vitro, which bind to chromaffin granule and plasma membranes, joining them together to form the "pentalaminar" fusion complexes characteristic of the first step in exocytosis.

B. Chemiosmotic Regulation of Chromaffin Granule Lysis

Exocytosis from a number of cell types may proceed by osmotic lysis of secretory granules that are fused to the inner aspect of the plasma membrane. Extracellular anions and intracellular H^+, at least in the chromaffin cell system, may provide the osmotically active particles to energize this process. We have, therefore, studied the exact mechanism by which chromaffin granules pump in H^+ and control their internal pH. In order to determine the internal pH of chromaffin granules we have devised a method to set the internal pH equal to the external pH over the pH range 3-8. The method essentially consists of mixing granules with weak acids and bases and ionophores to bring $\Delta pH=0$. Using this method the NMR titration curve of the P_γ of internal ATP was determined and was found to be very different from that of either Na^+, Ca^{2+} or Mg^{2+} ATP

in free solution. The titration curve of the internal ATP showed two inflexions in a ratio of about 1:2 with pK_a values of 6.4 and 4.3, respectively. These correspond to the phosphate (in the sodium form) and the adenine ring pK_a's, respectively. Thus, the P_γ atom is experiencing the titration of the adenine ring as a result of the mode of packing into the chromaffin granules. The P_γ of intra-granular ATP is deshielded, possible by co-planar juxtaposition with the adenine ring, as found in the crystal structure of Na_2ATP. From the ^{31}P chemical shift of the P_γ atom in these chromaffin granules, and using our calibration method for the titration of the internal P_γ, we obtain a value for the internal pH of 5.7, consistent with other methods, whereas the titration curve for free ATP gives a different value. It is apparent from the internal ATP titration curve that at pH 5.7 the ATP is present in the protonated singly charged state, while the adenine ring is unprotonated. Thus, at this pH there is a minimum amount of charge on the internal ATP, presumably aiding its packing into the granules. Upon addition of external MgATP, at either external pH=6.34 or 7.31, the internal pH was found to shift from pH 5.7 to pH 5.4. Previous work in this field has operated on the explicit assumption that the titration curve of the free metabolite was adequate for calibration of internal pH. We have shown that this is not the case. Among the main conclusions from this work is the fact that catecholamines probably do not interact with ATP. Possible interactions of this sort has been assumed for many years on, in retrospect, really minimal evidence. We have also found that transport of H^+ into the granule, by an ATPase-dependent electrogenic mechanism, can be monitored in a non-destructive, non-interactive manner using the ^{31}P-NMR signal from endogenous ATP.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 51,000-21 CHB

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October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Study of Immunology of Blood Cell Deficiencies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. Raphael Shulman	Chief	CHB, NIAMDD
Other:	Frances Gendelman	Chemist	CHB, NIAMDD
	Ellen K. McGlade	Masters Candidate, Univ. of MD	CHB, NIAMDD
	Alice Becker	Medical Student on Elective	CHB, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Autoimmunity, drug antibodies, platelet antigens, isoimmunization, transplantation antigens, hepatitis, hemagglutination tests, thrombocytopenia, blood coagulation inhibitors.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To study the nature of immunologic reactions which result in formation of antibodies against autologous blood cells, and to determine the significance of immunity in development of "idiopathic" blood cell deficiency states. Of special interest are the biochemical reactions which result in formation of complexes between cells, antibodies, and drug haptenes and the physiologic processes which result in sequestration of cells with attached antibodies. In recent years our work on identifying inherited leukocyte and platelet isoantigens has led to a study of the significance of these antigens in hetero- and homo-transplantation. Immunologic techniques have been used to develop a number of clinically practical diagnostic tests for certain infectious diseases and drug addiction.

Methods Employed:

Techniques of quantitative immunochemistry, including preparation and physiocochemical characterization of purified antibodies and antigens, microanalyses for nitrogen, histamine, and alkaloid drugs, quantitative measurements of complement fixation, cellular agglutination and precipitin reactions, immunoelectrophoresis, methods of provoking antibody responses in man and animals, and isotopic and fluorescent labeling applied to antigens and antibodies. Tissue culture techniques, red cell agglutination and techniques and lymphocyte transformation tests, including radioautography. Methods of hemagglutination involving attachment of various antigens to erythrocytes by chemical coupling.

Publications:

1. Markovic, N.S., Lipkin, L.E., Markovic, O.S. and Wade, M.J.: Device for quantitative cytochemistry: A computerized scanning microdensitometer 'grain counter'. J. Histochem. Cytochem. 26: 792-802, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 51,001-21 CHB

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. Raphael Shulman	Chief	CHB, NIAMDD
Other:	Edwarda M. Buda	Clinical Associate	CHB, NIAMDD
	Frances Gendelman	Chemist	CHB, NIAMDD
	Ellen K. McGlade	Masters Candidate, Univ. of MD	CHB, NIAMDD

COOPERATING UNITS (if any)

R.E. Goldstein	Senior Investigator	CB, NHLBI
J. Schulman	Senior Investigator	NPMB, NICHD

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hemophilia, coagulation mechanism, clotting factor assays, clotting factor purification, platelet physiology, platelet metabolism, pharmacology of platelet-inhibitory drugs.

PHS-6040
(Rev. 10-76)

Project No. Z01 AM 51,001-21 CHB

Project Description:

Objectives:

Study of the reactions and interactions of coagulation factors in vitro and in vivo to define further the nature of the blood coagulation mechanism, to determine factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis, and to develop better forms of therapy for these diseases.

Methods Employed:

Methods of protein purification and characterization, techniques of enzymology applied primarily to proteolytic enzymes and their inhibitors, kinetic analyses of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactivity labeled substrates.

Publications:

1. Shulman, N.R., Pollard, H.B., Tack-Goldman, K. and Buda, E.: A new class of drugs that inhibit platelet release and aggregation. Transactions of the Assoc. of Amer. Physcians XCI: 104-117, 1978.

2. Lipson, L.C., Bonow, R.O., Capurro, N.L., Goldstein, R.E., Shulman, N.R. and Epstein, S.E.: Relative effects of aspirin on prostaglandin modulation of coronary blood flow and platelet aggregation. Am. J. Cardiol. 41: 425, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 51,002-06 CHB

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Mechanisms of Hormone and Transmitter Secretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Harvey B. Pollard	Senior Investigator	CHB	NIAMDD
Other:	Christopher J. Pazoles	Senior Staff Fellow	CHB	NIAMDD
	Carl E. Creutz	Staff Fellow	CHB	NIAMDD

COOPERATING UNITS (if any) Laboratory of Chemical Pharmacology, DTP, DCT, NCI
Biological Psychiatry Branch, NIMH
Laboratory of Theoretical Biology, NCI
Developmental Pharmacology Branch, NICHD

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	3.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Exocytosis of neurotransmitters, hormones and other cell secretory products is believed to occur by Ca^{++}-dependent fusion of secretory vesicles with the plasma membrane and by subsequent breakage (fission) of the barrier separating vesicle interior from the extracellular medium. We recently discovered a new protein, synexin, that mediates the calcium dependent fusion event, and we have now found that Ca^{++} causes the synexin molecules to assemble into cigar-shaped units which then associate side-by-side and end-to-end to form large para-crystalline arrays. We have previously shown that the chemiosmotic mechanism of chromaffin granule lysis may have useful predictive value for understanding the membrane-breakage or release process in exocytosis. We have now invented a method of exactly quantitating proton entry into granules by ^{31}P nuclear magnetic resonance spectroscopy, and in so doing have discovered that the ATP inside chromaffin granules (concentration ~0.15M) was in a conformation different from that of ATP in simple solution. In exocytosis from chromaffin cells we propose that intracellular ATP induces entry of H^+ into fused granules, and that Cl^- from outside the cell also enters the granule and thus causes the local fused granules to undergo outward breakage (fission) and release.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The object of our work has been to discover the molecular bases for regulation of cellular secretion of hormones and transmitters by exocytosis. Our approach has been to search for factors mediating calcium action, the formation of complexes between secretory vesicles and plasma membranes and the final breakage (fission) of this complex leading to the ultimate release event of exocytosis.

Methods Employed:

The methods we have employed include cell fractionation, ultracentrifugation, light and electron microscopy, enzymology, scintillation counting, light scattering, fluorescence spectroscopy and nuclear magnetic resonance.

Publications:

1. Pollard, H.B., Menard, R., Brandt, H.A., Pazoles, C.J., Creutz, C.E. and Ramu, A.: Application of Bradford's protein assay to adrenal gland subcellular fractions. Anal. Biochem. 86: 761-763, 1978.

2. Pollard, H.B., Shindo, H., Creutz, C.E., Pazoles, C.J. and Cohen, J.S.: Internal pH and state of ATP in adrenergic chromaffin granules determined by ^{31}P-NMR spectroscopy. J. Biol. Chem. 254: 1170-1177, 1979.

3. Creutz, C.E., Pazoles, C.J. and Pollard, H.B.: Self-association of synexin in the presence of calcium: Correlation with synexin-induced membrane fusion and examination of the structure of synexin aggregates. J. Biol. Chem. 254: 553-558, 1979.

Annual Report Summary of the
Genetics & Biochemistry Branch, NIAMDD

Dr. Neufeld is continuing the study of lysosomal function in patients with genetic diseases (mucopolysaccharidoses and mucolipidoses) as well as in normal individuals. The biosynthesis of lysosomal enzymes in intact fibroblasts has been shown to be a complex process, requiring not only glycosylation and phosphorylation, but also proteolytic processing. As a prelude to purification of the receptor for lysosomal enzymes, a technique has been developed to directly measure the enzyme-receptor binding. Mutants of lysosomal enzymes have been isolated in CHO cells (Z01 AM 52000-12 GBB).

Drs. Zasloff and Camerini-Otero have undertaken a study of the biochemical basis of cystic fibrosis. One aspect of this project focuses on the selective advantage of mucoid strains of pseudomonads in the bronchial infections characteristic of this disease (Z01 AM 52004-01 GBB).

In the area of basic genetics, Drs. Camerini-Otero and Zasloff are continuing their work on gene expression in eukaryotes. They have found that the frequency of first cuts of DNA fragments by pancreatic DNase is much greater if the fragments contain an active gene. They have also introduced specific genetic material (human, chicken and Herpes thymidine kinase genes) into cultured mouse cells, and have shown that the foreign genes become integrated into mouse DNA. The nature of the integration is under study (Z01 AM 52001-02 GBB).

Dr. Zasloff is investigating the structural difference between genes encoding rather similar RNA's: $tRNA^{met}$ which is the common amino-acyl carrier, and $tRNA^{met}$ which initiates protein synthesis (Z01 AM 52003-01 GBB).

Dr. Camerini-Otero is continuing the study of cell-surface antigens by biochemical techniques. Proteins corresponding to the H2-L and H2-D mouse antigens have been separated by two-dimensional gel electrophoresis (Z01 AM 52002-02 GBB).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 52002-02 GBB Formerly: Z01 AM 41033-01 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Role of Cell-Surface Antigens in Development

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Rafael Daniel Camerini-Otero Senior Surgeon GBB NIAMDD
OTHER: Teresa Krakauer Staff Fellow GBB NIAMDD

COOPERATING UNITS (if any)

Transplantation Biology Section, Immunology Branch, NCI

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, National Institutes of Health, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.85	0.85	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been proposed that a male-specific histocompatibility antigen, the H-Y antigen, is responsible for gonadal sex determination in mammals, and perhaps all vertebrates. The proposal is based on immunologic and genetic data suggesting that the H-Y antigen is a testis-determining factor. Using the technique of two-dimensional gel electrophoresis we have been unable to find any evidence for such an antigen.

In a related set of experiments, we have found consistent differences in the two-dimensional electrophoretic behavior of three antigens of the mouse H-2 major histocompatibility complex (MHC), H2-K, H2-D and H2-L.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To understand the biochemical mechanisms by which cell-surface antigens are involved in mammalian differentiation and organogenesis.

Methods Employed:

Standard cell biology and biochemical techniques. Two-dimensional gel electrophoresis. Several serologic assays for anti-HY antibodies.

Major Findings:

1. Two-dimensional gel electrophoretic analysis of ^{35}S-methionine labeled NP-40 whole cell extracts of male and female syngeneic mouse spleen cells has not revealed any consistent differences. These are cells that by immunologic criteria contain the H-Y antigen.

2. Cell surface proteins from these cells were prepared by immunoprecipitation with goat antibody to whole male and female mouse spleen cells. When these proteins were examined on 2-D gels there were no differences between male and female cell surface proteins.

3. A technique has been developed for immunoprecipitating glycoproteins using a sandwich technique. The NP-40 ^{35}S-methionine labeled extract is first incubated with Concanavalin A, then staphylococcus aureus coated with goat anti-Con A antibody is added. The technique is specific for glycoproteins. When these proteins are examined on 2-D gels, they represent almost the same population of proteins that are immunoprecipitated with goat antibody to whole cells. Furthermore, there are no differences between the Con-A binding proteins of NP-40 extracts from male and female spleen cells.

4. We have also used the technique of two-dimensional gel electrophoresis to examine the polypeptides corresponding to the mouse MHC antigens. Recent evidence from Ted Hansen and David Sachs (I NCI) among others, has indicated that in addition to well-known H2-K and H2-D genetic loci and their corresponding antigens, there is probably a third loci in this complex, designated now as H2-L. Genetic, immunologic and some biochemical data indicate that the products of H2-L and H2-D are very closely related. We have recently been able to reproducibly distinguish them on the basis of 2-D gels.

Significance to Bio-Medical Research and the Program of the Institute:

This project will lead to a better understanding of: (a) the molecular mechanisms of differentiation and organogenesis; and, (b) the role of cell-surface antigens.

Proposed Course:

1. To use anti-HY antibody obtained from hybridomas (now being prepared in other laboratories) to immunoprecipitate and characterize the antigen.

2. To establish whether the electrophoretic differences between H2-D and H2-L are inherent in the polypeptide or are post-translational.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52004-01 GBB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Molecular Basis of Cystic Fibrosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	Michael A. Zasloff	Senior Surgeon	GBB NIAMDD
	R. Daniel Camerini-Otero	Senior Surgeon	GBB NIAMDD
OTHER:	Carmen Tejedor	Guest Worker	GBB NIAMDD

COOPERATING UNITS (if any)
Section on Enzymes and Cellular Biochemistry, LBM, NIAMDD
Cystic Fibrosis Center, Children's Hospital National Medical Center, Washington, DC

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, National Institutes of Health, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.7	0.05

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The molecular basis of cystic fibrosis, the most common lethal genetic disease of the Caucasians, is totally obscure. Two aspects of the disease are being studied: (1) the peculiar association of the mucoid variants of pseudomonas aeruginosa with CF patients, a bacteriological association almost pathognomonic of CF, and (2) the existence of low molecular weight membrane-active polypeptides with ionophore-like activity identified in fluids such as urine, serum, and the medium of CF fibroblasts in tissue culture.

3-3-0040
(Rev. 10-76)

Project Description

Objectives:

1. To understand the mechanism of mucoid-conversion in pseudomonads and to clarify the remarkable predilection of this species for the bronchial mucosa of people with CF.

2. To identify the ionophore-like activities reported to exist in CF patients by the development of a specific ionophore assay.

Methods Employed:

1. Growth and cultivation of pseudomonads; phage typing of pathological strains; isolation and characterization of mutant strains; quantitative assays of phage-bacterial interactions; quantitative growth rates of mucoid and non-mucoid variants; analysis of membrane components of pseudomonas; fractionation of human serum, sputum and urine.

2. Tissue culture, ionophore assays, plating efficiency, ionic component dependence, quantitative growth curves, permeabilization of mouse L-cells.

Major Findings:

1. We have confirmed the prevalence of the mucoid variants that inhabit the patient with CF. In vitro these strains are unstable with reversion rates that vary from hours to weeks depending on the isolate.

2. We have typed suitable isolates of mucoid and non-mucoid variants and confirmed their common genetic background.

3. We have demonstrated that lytic bacteriophage can effect powerful selection of mucoid over non-mucoid organisms.

4. We have isolated several mutants of both mucoid and non-mucoid resistant to 6-fluorotryptophan, 4-fluorotryptophan, methylglyoxal and azide.

5. Quantitative growth curves of both mucoid and non-mucoid variants have been established in a synthetic minimal medium as well as in normal salivary secretions.

6. Stocks of pseudomonad phages have been propagated and antiserum production has been initiated for use in the assay of phage-bacterium interactions.

7. The requirements for calcium ion have been determined for mouse L-cells (TK^-, $Aprt^-$) for both adhesion and growth. Ionophore-mediated transport of calcium at subminimal concentrations using A23187 has been demonstrated using cell proliferation as index.

Significance to Biomedical Research and the Program of the Institute:

1. Our studies have been initiated to attempt to understand the molecular defect of a common genetic disorder.

The studies should extend our knowledge not only of the defect in CF but of the peculiar features of the disease process that promote the inhabitation by a uniquely pathogenic bacterial organism and result in considerable morbidity.

2. The identification of ionophoric activity has been reported in diverse systems all marked by considerable irreproducibility. The establishment of a dependable assay system using proportional response should aid in the ultimate identification of the activity.

Future Course:

1. We will establish the basis of the prevalence of mucoid pseudomonas conversion in CF. We will pursue the problem assuming that such an association arises by one of the following mechanisms:

a. Growth advantage of the mucoid species in the milieu of the CF respiratory tract.

b. Stability of the genotype in the CF host.

c. Preferential selection or conversion by mechanisms other than growth advantage.

2. The growth of the mucoid versus non-mucoid organisms in CF fluids is being studied by quantitative procedures. Specific mutant stocks permit distinction between our laboratory strains from endogenous bacteria.

3. Since the mucoid phenotype is unstable in vitro, we will determine if CF fluids stabilize the phenotype by incubation of our defined strains in such fluids.

4. Although the genetic basis of mucoidy is obscure, its selection may be induced by the action of agents such as bacteriophage and aeroginocins. We wish to determine by analysis of CF fluids whether components that promote the selection of mucoid variants, perhaps by selection of phage-resistance, can be identified.

5. We plan to identify a possible ionophore-like activity in CF by assay of CF fluids for components that rescue mouse L-cells unable to grow in subminimal concentrations of calcium.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52000-12 GBB Formerly: Z01 A1 41024-11 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Biochemical Lesions in the Genetic Mucopolysaccharidoses and Mucolipidoses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	Elizabeth Neufeld	Biochemist & Branch Chief	GBB NIAMDD
OTHER:	Amos Frisch	Visiting Fellow	GBB NIAMDD
	Clara Hall	Research Chemist	GBB NIAMDD
	Andrej Hasilik	Guest Worker	GBB NIAMDD
	Evelyn Karson	Guest Worker	GBB NIAMDD
	Rachel Myerowitz	Guest Worker	GBB NIAMDD
	April Robbins	Staff Fellow	GBB NIAMDD
	Leonard Rome	Staff Fellow	GBB NIAMDD
	James Scott	Biologist	GBB NIAMDD

COOPERATING UNITS (if any)
Ultrastructural Pathology Section, LP, NCI
Section on Intermediary Metabolism, LBM, NIAMDD

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, National Institutes of Health, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9.5	8.5	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The biosynthesis of lysosomal enzymes in cultured human fibroblasts has been studied by labeling the cultures with radioactive leucine, mannose or phosphate, immunoprecipitating the radioactive enzymes and examining them by fluorography after electrophoresis in sodium dodecyl sulfate. Three enzymes, β-hexosaminidase (A and B isozymes), α-glucosidase and cathepsin D were found to be synthesized as precursors of larger molecular weight and subsequently trimmed to the size of tissue enzymes. In normal cells, the three lysosomal enzymes were found to contain phosphate as mannose 6-phosphate residues; phosphorylation was deficient in enzymes produced by fibroblasts of patients with I-cell disease. The mannose 6-phosphate constitutes a portion of the recognition marker for receptor binding and pinocytosis. By a novel screening technique, mutants of lysosomal enzymes have been isolated in CHO cells and used to demonstrate an uptake system recognizing mannose 6-phosphate, similar to that of human fibroblasts.

Project Description:

Objectives:

To study normal lysosomal function as well as the biochemical defects in genetic disorders of such function, as in mucopolysaccharidoses and mucolipidoses, and to use the findings for medical assistance to the patients and their families.

Methods Employed:

1. The binding of "high uptake" urinary α-L-iduronidase to intact fibroblasts has been measured. There are about 14,000 receptors per cell; $K_d = 10^{-9}$M; mannose 6-phosphate inhibits competitively with $K_i = 10^{-4}$M. During pinocytosis, the enzyme is delivered to lysosomes, whereas the receptors appear to recycle with a transit time of ca. 5 minutes.

2. Three lysosomal enzymes, β-hexosaminidase, α-glucosidase and cathepsin D were shown to be synthesized as precursors of larger molecular weight and subsequently processed to the size of tissue enzymes. This was established by labeling intact fibroblasts with [^{3}H]leucine, isolating the enzymes by immunoprecipitation, separating the polypeptide chains by gel electrophoresis in SDS and visualizing the radioactive chains by fluorography. The size difference between precursor and product was considerable in some cases (e.g., 63,000 to 29,000 for the β-chains of β-hexosaminidase) and less striking in others (e.g., 67,000 to 54,000 for the α-chains of the same enzyme). The secreted form of the enzyme was in the precursor form; there appeared to be no exocytosis of mature enzyme from lysosomes.

3. The same three hydrolytic enzymes were shown to be phosphorylated in normal cells, but not in cells from patients with Mucolipidosis II (I-cell disease). By use of $^{32}P_i$ and [^{3}H]-mannose and acid or enzymatic degradation of precursor polypeptides, the phosphate was shown to be present as mannose 6-phosphate on asparagine-linked oligosaccharide chains. Mannose 6-phosphate had been shown to be a key part of the recognition marker for pinocytosis. This confirms previous predictions of a defective recognition marker in mucolipidosis II and implicates the marker in the insertion of endogenous enzymes into lysosomes.

4. A "neoglycoprotein", bovine serum albumin to which 20 residues of aminophenyl mannose 6-phosphate had been linked, was a competitive inhibitor for α-L-iduronidase uptake. In addition, it was taken up by human fibroblasts and rapidly degraded. This uptake was blocked competitively by mannose 6-phosphate. Certain discrepancies in the kinetics of uptake of enzyme and glycoprotein suggest that the mechanism for the receptor-mediated pinocytosis of the two macromolecules is overlapping but not identical. Interestingly, uptake of the neoglycoprotein was completely absent in fibroblasts transformed by SV40.

5. A procedure has been developed for isolating mutants of lysosomal enzymes in CHO cells. Colonies on a culture dish are replicated on a filter paper, which is then used for measurement of enzymatic activity (by incubation in fluorogenic substrate and subsequent photography under UV light). Colonies which on the filter paper replica are positively stained for protein but not for enzyme activity are picked from the master dish and purified by cloning. Mutants for lysosomal α-mannosidase, β-galactosidase and α-L-iduronidase have been isolated in this manner. They were then used for study of enzyme uptake; the CHO cells were found to have a mannose 6-phosphate recognition system similar to that of human fibroblasts.

Significance to Biomedical Research and the Program of the Institute:

This project, begun in 1967, has led to the elucidation of biochemical defects and to the development of precise diagnostic methods for a class of genetic diseases. Current efforts deal with mechanisms responsible for the normal functioning of lysosomes and the correct insertion of hydrolytic enzymes into these organelles. The existence of a receptor-recognition system for hydrolytic enzymes suggests the presence of associated regulatory mechanisms, diseases of the receptor system, as well as the possible exploitation of this system for drug delivery.

Proposed Course:

1. The biosynthesis of lysosomal enzymes will be examined with respect to the processing of the carbohydrate portion and better structural analysis of the phosphorylated oligosaccharide.

2. Cultures of fibroblasts from patients with mucolipidosis II (I-cell disease) and the biochemically related though clinically milder Mucolipidosis III, and of heterozygotes, will be examined for the defect in phosphorylation. Is there heterogeneity in these disorders? Is the defect in phosphorylation primary, or is it secondary to some underlying defect in glycosylation?

3. The enzymatic steps involved in the phosphorylation of the enzymes will be sought in extracts of normal cells, and the corresponding enzymatic defect in cells of mucolipidosis II and III patients.

4. Mutants of CHO cells defective in uptake will be selected and analyzed. Those which have a defective receptor will be studied to determine the consequence on the insertion of endogenous enzymes into lysosomes.

5. The generality of the receptor-recognition system for lysosomal enzymes will be examined. Do cells other than fibroblasts (e.g., hepatocytes, Kupffer cells, leukocytes) produce phosphorylated lysosomal enzymes? If not, do they have a different marker for incorporating enzymes into lysosomes?

6. The receptor for α-L-iduronidase will be solubilized, purified and used to prepare antibodies. The consequences of growing cells in the presence of antibody to the uptake receptor will be examined. Intracellular receptors will be sought and their properties compared to those of the surface receptor.

Publications:

1. Sando, G.N., Titus-Dillon, P., Hall, C.W. and Neufeld, E.F.: Inhibition of receptor-mediated uptake of a lysosomal enzyme into fibroblasts by chloroquine, procaine and ammonia. Exptl. Cell Res. 119:359-364 (1979).

2. Robbins, A.R.: Isolation of lysosomal α-mannosidase mutants of Chinese hamster ovary cells. Proc. Natl. Acad. Sci. USA 76:1911-1915 (1971).

3. Rome, L.H., Weissmann, B. and Neufeld, E.F.: Direct demonstration of binding of a lysosomal enzyme, α-L-iduronidase, to receptors on cultured fibroblasts. Proc. Natl. Acad. Sci. USA 75:2331-2334 (1979).

4. Rome, L.H., Garvin, A.J., Allietta, M.M. and Neufeld, E.F.: Two species of lysosomal organelles in cultured human fibroblasts. Cell 17:143-153 (1979).

5. Nwokoro, N. and Neufeld, E.F.: Detection of Hunter heterozygotes by enzymatic analysis of hair roots. Am. J. Hum. Genet. 31:42-49 (1979).

6. Frisch, A. and Neufeld, E.F.: A rapid and sensitive assay for neuraminidase: application to cultured fibroblasts. Anal. Biochem. 95:222-227 (1979).

7. Neufeld, E.F.: The uptake of enzymes into lysosomes - an overview. Enzyme Therapy in Genetic Diseases, National Foundation - March of Dimes, in press.

8. DiNatale, P. and Neufeld, E.F.: The biochemical diagnosis of mucopolysaccharidoses, mucolipidoses and related disorders. Proceedings of the Second National Congress of Inborn Errors, Milan, Italy, (1977) in press.

9. Neufeld, E.F. and Ashwell, G.: Carbohydrate recognition systems for receptor-mediated pinocytosis. In "Biochemistry of Glycoproteins and Proteoglycans" (W.J. Lennarz, ed.), Plenum Press, in press.

10. Shapiro, L.J. and Neufeld, E.F.: Genetic mucopolysaccharidoses and mucolipidoses. In "Basic Neurochemistry" (R.W. Albers, G.J. Siegel, R. Katzman and B.W. Agronoff, eds.), Third Edition, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52001-02 GBB Formerly: Z01 AM 41032-01 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	Rafael D. Camerini-Otero	Senior Surgeon	GBB NIAMDD
	Michael A. Zasloff	Senior Surgeon	GBB NIAMDD
OTHER:	Teresa Krakauer	Staff Fellow	GBB NIAMDD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, National Institutes of Health, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.45	1.85	0.60

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To understand the control of gene expression in higher eukaryotes we must be able to: (a) easily distinguish active genes from inactive genes at the level of the nucleus, and (b) to introduce genes with well-defined DNA sequences into cells and observe their expression. Experiments have been performed using both approaches.

Pancreatic DNase has been successfully used to monitor the first-hit nucleolytic attack of genes in nuclei. Using this technique active genes can be easily distinguished from inactive genes.

Also, we have been able to successfully introduce several genes into mouse Ltk⁻ cultured cells. The introduced genes are integrated into DNA carried in these cells and some are actively expressed, a process designated as biochemical transformation. The nature of this integration and expression is now being actively investigated.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To study the molecular control of gene expression. To use this knowledge to develop new techniques for gene purification.

Methods Employed:

Methods of molecular biology and biochemistry. Recombinant DNA methodology. Techniques of cell culture and somatic cell genetics.

Major Findings:

1. Pancreatic DNase has previously been shown to digest the DNA of active genes more quickly than the DNA of inactive genes. We have been able to show that an even more sensitive assay for active genes is to measure the frequency of first cuts or nicks of the DNA within defined fragments of increasing size containing the gene. Restriction endonuclease fragments containing the β-major chicken globin gene were prepared from chicken erythrocyte nuclei digested with increasing amounts of pancreatic DNase. "Southern blots" of these double digests were then carried out against a cDNA clone of the globin gene. The rate of disappearance of the fragments is then directly related to the frequency of first cuts of the gene by the DNase. Using this technique the frequency of first cuts is about 30 times higher for an active gene (globin) compared to an inactive control gene (albumin).

2. Using the technique described above we have also mapped the extent of the chromatin conformational alteration responsible for the increased DNase sensitivity. The results indicate that the increased rate of first cuts does not extend from the active gene to nearby sequences. For example, a 9 kb restriction fragment containing the globin gene and flanking sequences, sustains first cuts at the same rate as a 1.3 kb fragment containing only the globin gene.

3. We have been able to introduce genes into mammalian cells. The cells used as recipients have been mouse L cell mutants for biochemically selectable markers: Ltk$^-$ (lacking thymidine kinase, tk), Ltk$^-$ Aprt$^-$ (lacking tk and adenine phosphoribosyl transferase, Aprt), A9 (lacking hypoxanthine-guanine phosphoribosyl transferase, Hgprt) and a line selected by us Ltk$^-$ Aprt$^-$ Hgprt$^-$. So far, the only well-characterized transformants have been obtained with the Ltk$^-$ Aprt$^-$ cells. Genes that have been introduced into this line (which is nonreverting for the tk$^-$ mutation) have been the Herpes simplex virus type I, thymidine kinase gene, and the human and chicken thymidine kinase genes. The frequency of transformation with the purified 3.4 kb BAM H1 fragment containing the Herpes tk gene is about 1 transformant per 25 to 50 picograms per 10^6 cells.

The frequency using total unfractionated human or chicken DNA is about 1 transformant per 10 to 20 micrograms per 10^6 cells. Several of the Herpes tk transformants have been mapped with restriction endonucleases. In all of them, the fragment bearing the tk gene has integrated into DNA being carried by the cells.

Cotransformation experiments have been carried out with the cloned Herpes tk gene and the DNA of Charon phage (a lambda phage derivative), and pBR322 plasmid. Transformation has also been carried out with the Herpes tk fragment ligated to pBR322. These transformants are now being mapped.

Attempts to transform the A9 cells ($Hgprt^-$) with human DNA have so far only yielded revertants bearing the mouse Hgprt. Whether this event, which appears to occur at a higher frequency that reversion, is in fact mediated by DNA is being investigated.

Significance to Biomedical Research and the Program of the Institute:

In order to understand normal human development, developmental anomalies and genetic disorders we must first understand the mechanisms controlling the expression of genes in mammalian cells, the latter is the purpose of much of the work being carried out. Furthermore, the ability to introduce genes into cells might enable us to purify genes that cannot be isolated with the present technology. Many of the genes for known human genetic disorders are of this type.

Proposed Course:

1. To extend the use of pancreatic DNase to other genes, particularly inducible systems, such as the genes that are activated by hormones. Such systems should allow us to study the sequence of nuclear events resulting in transcription.

2. To modify genes in vitro and then introduce these into cells. The objective here is to study how changes in the DNA affect the expression of the gene and of nearby sequences.

3. To investigate the nature of the integration events in biochemical transformants. For example, are transferred genes in nucleosomes or not? Is the packaging of genes (in nucleosomes, for example) dependent on the state of activity of the gene? Is pancreatic DNase a good probe for gene activity in these newly introduced genes? What is the nature of the flanking sequences after the integration event, for example, are there special cellular sequences which readily accept exogenous genes? Are the integrated sequences truly chromosomal or have they integrated in a replicating episome?

4. To devise a gene purification scheme using biochemical transformation as an assay for the biologically active sequences of a gene.

Publications:

Simon, R.H., Camerini-Otero, R.D. and Felsenfeld, G. (1978) An octamer of histones H3 and H4 forms a compact complex with DNA of nucleosome size. Nucleic Acids Research 5:4805-4818.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52003-01 GBB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structure of Genes for Methionyl tRNA in the Human Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	: Michael A. Zasloff	Senior Surgeon	GBB NIAMDD
OTHER:	Tomas Santos	Visiting Fellow	GBB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, National Institutes of Health, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.8	0.10

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We wish to explore differences in the structure of two human tRNA genes, one coding for the $tRNA^{met}$ which functions as the general initiator for eukaryotic protein synthesis and the other for the $tRNA^{met}$ serving as a standard amino-acyl carrier. The genes are being isolated from a recombinant population of human fetal liver DNA cloned in phage Charon 4A. Screening of the "library" is accomplished by Benton-Davis technology using nucleic acid probes generated from cloned Xenopus $tRNA^{met}$ fragments, ^{32}P-labeled rabbit tRNA, and ^{35}S-methionyl-tRNA. It is our expectation that basic structural features will distinguish two very similar gene products with totally divergent functions.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1. To understand the structural features characteristic of the genes coding for methionine tRNA acceptors in the human genome.

2. To define features which distinguish the two functionally distinct methionine tRNA acceptors at the level of gene structure.

3. To understand the regulation of expression of these gene products.

Methods Employed:

Nucleic acid hybridization using cloned cDNA and genomic sequence probes; Benton-Davis phage screening techniques; southern transfers; DNA cloning using Charon phage and EK2 plasmid rectors; nick translation of probe DNA.

Major Findings:

1. A library of human fetal liver DNA has been constructed from a partial Hae III and ALu I restriction nuclease digest cloned in the propagating phage Charon 4A. The library represents a population of about 3×10^5 random fragments.

2. The library has been grown using multiple soft agar surface cultures to maintain stock stability through a 10^5-fold amplification.

3. A set of methionyl tRNA probes has been generated. ^{35}S-methionyl tRNA, specific for both the initiator and the internal species have been synthesized using partially purified amino acid tRNA ligases from rabbit liver and E. coli.

4. For a tRNA probe permitting the detection of the entire set of genomic fragments containing tRNA sequences, a $5'$-^{32}P-labeled tRNA preparation has been synthesized using T4 polynucleotide kinase and rabbit-liver tRNA.

5. A specific DNA fragment, homologous to the human $tRNA^{met}_i$ sequence has been isolated from a X. laevis genomic fragment cloned in pBR322 by digestion with Hinf I. The 183 base pair fragment has been nick-translated and is used as a probe for the initiator sequence in the human library.

Significance to Biomedical Research and the Program of the Institute:

1. The project attempts to understand the molecular basis of specific gene expression in the human genome.

2. With the genomic probes that will be isolated, fine structural analysis or a specific human gene, not previously studied in such detail, will be achieved.

Proposed Course:

1. The library of human fetal liver DNA is being screened for all detectable fragments containing methionyl tRNA sequences.

2. The segments will be mapped and then sequenced using restriction nuclease digestion and direct nucleotide sequencing techniques.

3. Correlations between genetic and tRNA sequences will be made, attempting to identify processing events including RNA splice points, base modifications, and RNA additions.

4. With the specific human genomic fragments at hand, probes will be available for both the specific tRNA's as well as neighboring sequences. Questions such as linkage, chromosomal loci, cell-cycle timing of tRNA synthetic events, precursor structure, and the nature of flanking sequences will be asked.

5. Correlations of the functions of specific portions of the genes will be explored in in vitro systems, a totally reconstituted system employing histones and RNA polymerase III as well as the X. laevis germinal vesicle system.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 52005-01 GBB Formerly: Z01 AM 41034-01 ARB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (30 characters or less)

Eukaryotic Gene Expression and Cytoplasmic-Nuclear Exchange

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael A. Zasloff Senior Surgeon GBB NIAMDD

COOPERATING UNITS (if any)

None

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION

INSTITUTE AND LOCATION
NIAMDD, National Institutes of Health, Bethesda, Maryland 20025

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

INACTIVE

PHS-6040
(Rev. 10-76)

Office Of The
Associate Director For
Extramural Activities Program

The most significant development in FY 1979 was the addition of the Contract Office to the Extramural Activities Program. This unit was formerly part of the Executive Office. It is envisioned that the addition of this unit will facilitate operations of our contracting efforts. There was no further expansion of the staff of the Extramural Activities Program and FY 79 was devoted primarily to consolidating the personnel gains of FY 78. The immediate office of the Associate Director saw the addition of a Special Assistant who will provide operational and technical support in program analysis and administrative services.

The Institute signed agreements with the Division of Research Resources and the National Institute of General Medical Sciences which implemented our participation in their respective Minority Biomedical Support (MBS) and Minority Access to Research Career (MARC) programs.

Grants Management Branch

A major thrust in the area of Grants Management occurred during this fiscal year. Both the Department and PHS placed additional emphasis on financial evaluation of grant proposals. Specifically this placed additional requirements on our staff to perform cost and price analysis on grant budgets. To that end, a workshop was offered by PHS to train Grants Management Specialists in this area. This course was attended by our staff including the GMO's. While it is recognized that the application of this training will result in a better managed grant program, it will also result in the need for a more detailed and lengthy review by branch personnel.

During fiscal 1979, the Department announced a goal of obligating not more than 30% of grant and contract dollars during the fourth quarter (July 1, 1979-September 30, 1979). An analysis of this Institute's award distribution indicates that we will be "hard-pressed" to meet this requirement. In order to comply with this directive and to move toward the goal of issuing awards with prospective begin dates we are discussing the feasibility of using facsimile signatures on awards. It has been determined by the NIH legal staff that such signatures are acceptable on award documents. While we do not know what final decision will be made by the Institute, our proposal is that both the Grants Management Officer's and the Program Director's signatures be affixed by facsimile signature stamps. Further, we are proposing that these signatures be affixed by personnel of this branch's Award Unit. This change will save 1 to 2 days processing time and will also eliminate the need for staff to transport the files between Grants Management and Program Offices.

Although we have recently recruited 2 additional Grants Management Specialists to the branch, we remain understaffed in terms of our ability to adequately carry out the full range of duties assigned to the branch. This is made especially true because of PHS and Department policies that assign additional

functions to Grants Management. This year, as indicated earlier, the additional function is a requirement for a more in-depth cost and price analysis of grant budgets. Further, we are currently woefully short of clerical help to the extent that large amounts of overtime are required to complete the work of the branch. It is hoped that we will be allowed to fill these vacant positions (3) during the remainder of the year. An offshoot to the problem of adequate personnel is the problem of adequate space. While the pending renovation plan (when accomplished) will help to alleviate the crowded conditions and will prvode for needed separation between people, one current aspect of separation is increasingly unworkable. That is, the fact that a small portion of the brnach (2 people) are located in space on another floor which causes problems in terms of work flow and in proper supervision.

Operations Office

We are still faced with the inadequate space problem that we have had since we moved two years ago and are now hoping to get a professional space consultation. We have, however, been able to move some of the inactive and closed-out files to another area thus relieving the critical file shelf situation.

We are again recruiting for a second person in the council preparation unit as our former employee joined the Apprenticeship Training Program.

Contracts Management Branch

The Contracts Management Branch (CMB) now reports to the Associate Director for Extramural Activities Program instead of the Executive Officer. Physical relocation from the NIH reservation to the Westwood Building was also effected.

Partially as a result of issuance by DHEW of their final audit report on the Institute's contracting activities, a task force was assigned to develop guidelines for NIAMDD contracting procedures. The document is comprehensive; input was also acquired from the Division of Contracts and Grants. It should prove to be of value to program managers, project officers, and executive secretaries.

Annual obligations under the contract mechanism - based on the best available projection to date - should equate to 15 million dollars during this fiscal year.

Heavy emphasis continues to be placed on administrative close-out of physically completed contracts. The Secretary's initiatives to correct alleged deficiencies have not abated. Instead, accelerated and sometimes unrealistic goals are being continually mandated. Most severely impacted are noncompetitive procurements (both new awards and renewals) and curtailment of traditionally heavy fourth quarter funding activity. Each mandate is replete with onerous reporting requirements that infringe upon our already inadequate manpower resources.

Again this year, no gain in personnel has been achieved since decentralization of contracting authority in 1971. In contrast, annual obligations have tripled. Adequate office space cannot be obtained. Inadequate space in combination with

the heavy workload yields predictable results - deterioration of quality and professionalism.

Lead time to complete awards lengthens with each additional administrative burden, such as prior approval/clearances, pre-solicitation reviews, pre-award reviews, ad infinitum.

The prospect of using cooperative agreements extensively in lieu of contracts does not appear realistic. Many problems remain to be resolved in regard to defining acquisition versus assistance, disposition of patent rights and other proprietary information and/or trade secrets. Commercial participation by fee bearing profit organizations may also further cloud the issue.

Program Evaluation Branch

A review of PEB's operations during the past nine months reveals additional workloads; a major accomplishment in streamlining the computerized system; increased productivity by staff; and no increase in full-time staff.

Over 4000 applications referred to the Institute including non-competing renewals were assigned program areas and most were scientifically coded. More than 60 formal requests for information were answered, many of these were concerned with trans-NIH issues. Some examples are noted below:

- Federal Inventory of Population Research - abstracts of 43 NIAMDD grants were included.

- NIH Inventory of Clinical Trials for FY's 77 and 78. NIAMDD reported 48 trials for FY 77 and 51 for FY 78.

- NIH report on Basic, Applied and Development (BAD) activities and Science Base, Applied, Transfer and Training (SATT) activities. The PEB coordinated the extramural program responses.

- Diabetes Mellitus Coordinating Committee (DMCC) for subtopics on all research related to diabetes. PEB produced over 25 tabular reports for DMCC on diabetes activities in the NIAMDD.

- History of council actions in the kidney, urology program for the past two years.

- Special report on Interims funded in FY 78 and FY 79 to date, which shows a decrease in hardship supplements awarded in FY 79.

- Report covering 1972 - 1978 showing all grants supported by NIAMDD doing research on knee and hip implants, transplants (bone), prosthesis and implant materials.

New update and edit computer programs are being tested now. These will be operational within a short time and will move data from the IMPAC file to the AM information system on a routine basis and in the AM format. This will end our duplication of effort with the IMPAC system.

Two of the most widely used recurring reports have been tabulated using new computer programs and are available to staff. These are the commitment and the year to date funded reports by program area.

Although contract information is not in the AM system, in response to the Review Unit needs, we are providing them contract data from the IMPAC system. Also, we have designed a subfile to track responses to RFP's for the Unit.

In summary, we accomplished the critical goals of the branch, that is, those tasks necessary for the minimum needs of the Institute.

Additionally, a few new achievements were completed. However, the plans made last year for: incorporating contracts and interagency agreements into the AM system; more front-end programming; and more analyses of the information available, were not fulfilled. The main reason being that staff could not be increased. We had some relief using overtime and part-time workers, a Stride trainee and a new computer terminal. In order to maintain a creditable information system capable of meeting the foreseeable requirements of the Institute, we must have adequate permanent support staff.

Review Unit

In December 1978 the Artificial Kidney-Chronic Uremia Advisory Committee, a chartered committee established for peer review of contract proposals, was transferred to the Review Unit to function under the direction of staff of the Contract Section. A request for renewal of this committee is pending. At the present time this is the only chartered committee within the Review Unit. Reviews of contracts not handled by this committee and reviews of all grant applications are performed by ad hoc review groups. A request for chartering of three review committees for grant applications is also pending.

A major change in review procedures has been implemented with program project applications received for the January 1979 Council. The review of individual component parts of program projects by study sections has been discontinued. The complete review of program projects will be accomplished by peer review committees of the Review Unit. A special announcement in the May issue of the NIH Guide to Grants and Contracts advised the scientific community of this change. In keeping with this change, the administrative guidelines for applicants in submitting program project applications have been revised and are available to applicants upon request.

The review workload within the Review Unit for the fiscal year is reflected in the appended Table I. The workload profile for the Review Unit has not yet been sufficiently stabilized or characterized to permit projections for long term manpower needs. However, it is clear that our present staff needs additional resources to enhance their abilities to maintain a high standard of performance and to meet the deadlines within the time constraints imposed by the review cycles. A major step in this direction is the consideration of and subsequent request for word processors to be used by the Grants Assistants. In addition, a microfische reader has been located in the Review Unit to assist in developing background information necessary for reviews.

The microfische produced from the data bank of the Division of Research Grants are updated every two weeks and permit ready access for all staff to these data. The need to travel two floors for document duplication facilities should be eliminated by the pending request for appropriate equipment on the same floor as that of this and other units of the Institute.

Plans made for FY 1979 to strengthen the Review Unit by increasing its staff to 15 members were unable to be fulfilled. This would have provided each of the three Review Sections with two executive secretaries and two grants assistants, and the Receipt and Control Section with two clerk typists, one of whom would double as Senior Grants Technical Assistant under the Chief of the Review Unit. The total resource would have reflected 15 man years. Due to restrictions imposed by personnel ceilings, the Review Unit strength, at the time of this writing, lacks a commitment for 4.25 man years to meet the strength originally contemplated.

TABLE I. NUMBER OF PROPOSALS REVIEWED BY REVIEW UNIT.

	COUNCIL			
ACTIVITY CODE *	AUG '78	JAN '79	MAY '79	FY '79
P01	7	6	4	17
T32	44	7	20	71
P30,50, 60	80	6	6	92
N01	29	48	68	145
TOTAL	160	67	98	325

* P01 = Program Projects, T32 = Institutional Fellowship Applications
P30 = Core Center Applications, P50 = Specialized Centers of Research
P60 = Comprehensive Centers, N01 = Contract Proposals
N01 = Proposals reviewed from the first day of one Council month to that of the next Council month are assigned to the latter Council.

Administrative Office

In early FY 1979, with the further expansion of the Clusters, the administrative functions of the Extramural Programs were divided among the Clusters, leaving this office responsible only for the EAP Cluster. This resulted in servicing approximately 40% of the total EP staff it had been and, with the Administrative Office staff reduced by one clerk-typist at about the same time, this office continued to find its time well occupied. With the addition of the NIAMDD Contracting Office to the EAP Cluster and, consequently, review and management of contracts added to the Cluster functions, this resulted in a substantial increase in the Administrative Office providing services as related to travel. The introduction of the early phases of the Material Management System at the NIH has resulted in numerous changes in both the procurement functions and travel functions of the office. As this System continues to expand during the next several months, it is anticipated that the resulting complications, which are being introduced into the administrative offices, will occupy more and more time and effort. The availability of sufficient space for both staff, grant, and contract files persists as a severe problem for the EAP Cluster. Despite efforts at various levels in NIAMDD, it would appear that this problem will continue indefinitely.

ANNUAL REPORT

DIABETES, ENDOCRINOLOGY AND METABOLIC DISEASES CLUSTER

Office of the Associate Director

The Associate Director plans and directs programs of research grants and contracts, training and fellowship awards, and special programs and activities within the Diabetes, Endocrinology and Metabolic Diseases cluster. Emphasis is directed toward integrating these programs into a coordinated effort by focusing on the aims, goals and opportunities common to these program areas. Efforts are being made to strengthen existing, and initiate new, programs in accordance with Institute objectives.

One special activity in the Endocrinology and Metabolic Diseases Program in which the Associate Director is actively involved is the ongoing evaluation of the research needs and opportunities in endocrinology and metabolic diseases by the scientific community.

The diabetes activities of the DEMD cluster are actively growing and the Associate Director is intimately involved in the Institute's developing research, training and information transfer programs in this area. In addition, the Associate Director, acting through the Inter-NIH Diabetes Coordinating Committee has primary responsibility for the management of all trans-NIH activities relating to diabetes mellitus. This program assures better coordination, a sharper focus for program activity, and accountability in diabetes.

The Associate Director is Chairman of the Inter-NIH Diabetes Mellitus Coordinating Committee, the Inter-Agency Diabetes Mellitus Coordinating Committee and serves as an ex-officio member of the National Diabetes Advisory Board. He is a member of the research committee of the American Diabetes Association and is frequently and intimately involved with the activities of the Juvenile Diabetes Foundation, the American Diabetes Association, the Endocrine Society, the Cystic Fibrosis Foundation and other groups and agencies concerned with diabetes, endocrinology and metabolic diseases.

DIABETES PROGRAMS BRANCH

During the past year, this office was established in order to coordinate the activities of the various DEMD diabetes programs and to provide an administrative focus for extramural grant and contract support of special diabetes research initiatives. In this regard, areas of special research interest have included studies related to islet cell transplantation, insulin delivery systems, behavioral research and epidemiology.

With respect to the development of procedures for transplantation of insulin-producing pancreatic islet cells, one of the major problems encountered during the last several years has been that of allograft rejection. Recently, however, grantees of the program have developed new techniques which markedly prolong the survival of rat islet allografts across a major histocompatibility barrier. This demonstration now suggests that immune rejection may not prevent the development of islet transplantation as a successful therapy in animal diabetes, although a number of other significant problems remain before the potential clinical application of these procedures can be established. Specifically, it will be necessary to (1) define further the mechanism of immune rejection and prevent its occurrence so that islet cell transplantation can be successfully applied to human diabetes; (2) develop improved techniques for selective, mass isolation of beta cells from pancreatic tissue; (3) develop improved procedures for long-term storage of islet cells prior to transplantation; (4) evaluate further the relative advantages of various transplantation sites with regard to the specific survival and functional integrity of beta cells; (5) assess the impact of successful transplants on plasma glucose control and other indices of metabolic homeostasis in diabetes.

Another promising area of research interest is related to the development of improved devices for regulating the delivery of insulin to the diabetic. In this regard, two general types of mechanical devices are being developed. One of these, referred to as a "closed-loop" system, consists of a glucose sensor, an insulin pump, and a control circuit that regulates the rate of insulin administration as an on-line function of measured glucose. At the present stage of development, closed-loop systems are large, complex devices which require a substantial commitment of highly trained manpower and other specialized medical resources for use. In addition, such devices virtually immobilize a patient in a bed or chair and can therefore only be used on an inpatient basis for treatment of acute emergencies, or for short-term experimental studies. Further development of refined, miniature closed-loop systems is limited by a number of factors including the lack of a reliable and specific glucose sensor with physical and biological characteristics that would make it appropriate for long-term studies.

The other type of device, the "open-loop" system, essentially consists of an insulin pump and a control circuit that regulates the rate of insulin infusion according to a pre-programmed pattern. A number of prototype open-loop systems have been developed which are portable and offer the potential for use in long-term ambulatory studies. The program is providing increased support for the further refinement and evaluation of such systems which appear

to offer the potential for improving plasma glucose control in insulin-dependent diabetic patients to a greater degree than that achieved by traditional therapy. Nevertheless, fundamental questions remain, including (1) the need to assess alternate routes of insulin delivery, e.g., subcutaneous, intraperitoneal, intraportal and peripheral venous routes; (2) development of appropriate computer programs for regulating insulin delivery in a manner which maintains metabolic homeostasis; (3) the need to develop biocompatible systems which avoid problems such as inflammation, clotting, tissue reaction, infection and fever; (4) the problem of ensuring insulin stability during storage at room or body temperatures for prolonged periods in reservoirs/pumps/catheters composed of various foreign materials; (5) the need to refine available pumps to ensure safe, accurate, reliable operation during long-term use in ambulatory patients; and (6) the need to assess the impact of long-term use on metabolic homeostasis in the diabetic patient. It is anticipated, however, that with further development, these systems will provide a mechanism to advance our understanding of the pathophysiology of diabetes and as well, may offer the potential for future clinical applications.

Programmatic efforts to stimulate research interest in the behavioral and psychosocial aspects of diabetes continued throughout the past year. In this regard, the Director of the National Diabetes Information Clearinghouse coordinated development of a Program Announcement entitled "Behavioral Studies Related to Diabetes Mellitus". This announcement which was co-sponsored with several other NIH Institutes and the National Institute of Mental Health, particularly encouraged interdisciplinary studies at the interface between the biomedical and behavioral sciences. In addition, a National Conference on Behavioral and Psychosocial Issues in Diabetes was also sponsored to define opportunities and priorities for future research efforts. The recommendations from this conference will contribute to the development of future program plans in these and related areas.

With respect to epidemiology, recent programming efforts coordinated by the Director of the Diabetes Data Group have resulted in an increased number of grant applications proposing epidemiologic studies. Although the approval rate for these applications has been relatively low, six meritorious projects now have been initiated studying discrete populations in Australia, Israel, Massachusetts, Oklahoma and Pennsylvania. In addition, plans are now being developed for a Workshop on Epidemiologic Methods in an effort to further extend research capabilities in relevant disciplines.

Diabetes Research Program

With the exception of those areas noted above, this program supports the full range of basic and clinical research in diabetes through extramural, investigator-initiated research and program project grants. As such, studies in progress focus primarily on the nature, etiology, and pathogenesis of diabetes and its complications in an effort to provide an improved understanding of both the disease itself as well as its vascular and neuropathic sequelae. In this regard, some areas of particular research interest include

studies of (1) the biosynthesis, secretion, action, interaction and degradation of insulin and other hormones and factors associated with diabetes; (2) the physiology and pathobiology of pancreatic islet cells; (3) abnormalities in carbohydrate, lipid and protein metabolism associated with diabetes and its complications; and (4) the possible etiologic significance of various genetic, viral, immunologic and nutritional parameters. Ultimately, the knowledge gained from studies in each of these areas can be expected to contribute to the development of improved medical capabilities for prevention, diagnosis, treatment and cure of diabetes and its complications.

As suggested above, the regulation of insulin secretion represents an area of continued active research interest. Although glucose appears to be the primary physiological stimulus for insulin secretion, it has not been established whether a metabolite of glucose or metabolic co-factor or possibly glucose itself acts as the actual trigger for insulin secretion. Nevertheless, studies have demonstrated that moderate to high concentrations of glucose directly or indirectly stimulate a biphasic release of insulin, with a rapid initial peak followed by a slower secondary rise. It has been suggested that these two stages of insulin release may reflect distinct phenomena, each under separate, possibly converging regulatory control. Recently, repid-phase insulin release which appears to be defective in maturity onset diabetes, has been observed for the first time in culture systems of isolated islets which retain normal beta- and alpha-cell function. Synthesis of D-glucose analogs is now in progress for use in studies with this culture system to further examine the structural characteristics of the gluco-receptors which regulate the secretion of insulin and glucagon.

There is also evidence for a factor from the neuro-intermediate lobe of the pituitary gland which stimulates insulin secretion. This factor appears to be a corticotrophin-like peptide closely related to a fragment of ACTH. Results suggest that high insulin levels, partly under pituitary control, and high caloric intake may be interlinked and in combination, could be a major factor in the early development of gentic obesity in experimental animals. It has also been shown recently that the chronic administration of porcine ACTH produces a syndrome similar to that displayed by the ob/ob mouse (hyperglycemia and insulin resistance). There is a single gene mutation in this mouse and diabetes appears to be a relatively late consequence of the mutation. Insulin resistance does not develop until after hyperglycemia and glucagon and cortisone are elevated well before hyperglycemia.

Investigators of the mechanism of insulin action involves studies of both the binding of insulin to its target cells and the diverse effects of insulin on the cells. Considerable evidence suggests that insulin receptors located on the plasma membrane of target cells directly mediate the short-term effects of insulin on plasma membrane functions such as glucose transport. Insulin also exerts a large number of diverse effects in the cells and the timing of these events is important - some effects are immediate and some take hours to days. The discovery that insulin directly regulates its cell surface receptors has led to new insights into the mechanisms involved in several insulin-resistant and insulin-sensitive states. The mechanism

for insulin's regulation of long-term effects on the cell interior is still unknown. One theory is that insulin, like glucagon, generates a second messenger at the cell surface which subsequently is responsible for the intracellular effects of insulin. Another theory is that insulin itself enters the target cell and that it or a by-product interact directly with intracellular components. Recent studies support the theory that insulin does enter the cell and that intracellular organelles have specific binding sites for insulin. In this regard, one laboratory has reported evidence that isolated nuclei contain specific binding sites on the surface membrane.

The extent to which the mitogenic activity of insulin is due to interaction with its own receptor or with the receptors of NSILA's, somatomedin or MSA has not yet been determined. Nevertheless, understanding the mechanism of action as well as interaction of insulin and insulin-like peptides is of considerable significance. One laboratory is constructing hybrid cell clones from cell lines that have only one or the other of these receptors and are defective in subsequent actions. Another grantee is looking at the effect of growth promoting molecules on the process of cellular differentiation. It has been demonstrated recently that there is significant polyploidization in the B-cells of diabetic mice at 4-5 weeks of age when diabetic symptoms are emerging. It has been suggested that this phenomenon may be one of the earliest indicators of impending disease reflecting some basic cellular defect.

Animal models are extremely important as tools for diabetes research. In this regard, it is not necessary that any given model duplicate every aspect of the human disease. It is likely that the primary metabolic disturbance in diabetes will be demonstrated in an animal where genetic modifiers can be more easily defined than in man. During the past year, the program has continued to actively solicit research in the area of the characterization of such models. At the present time, studies are being supported to characterize at least twelve different animal models of diabetes, with primary emphasis on the ob/ob and db/db mice, the Chinese hamster and the BB Wistar rat.

The synthesis of insulin using recombinant DNA techniques represents another area of active research interest. In this regard, one of the first practical spin-offs of recombinant-DNA research has been the artificial creation of a form of rat insulin. More specifically, a team of scientists from Harvard University and the Joslin Diabetes Foundation used a copy of the rat's natural gene for induction of insulin synthesis in E. coli bacteria. This accomplishment was a forerunner of the subsequent production of a biological carbon copy of human insulin by a research team at the City of Hope National Medical Center in Duarte, California. If successfully developed, the new procedure holds potential for mass insulin production, alleviation of insulin allergy, reduced cost of treatment and assurance of adequate supplies of the hormone for the diabetic population. Recombinant DNA techniques also offer the opportunity to study the molecular basis for

glucose regulation of insulin secretion. Studies in progress have recently demonstrated that the translation efficiency of pro-insulin mRNA is extremely sensitive to slight changes in the translation system. However, the actual relationship between this observation and deficiencies in insulin secretion in vivo has not been determined.

With respect to the etiology of diabetes, genetic and epidemiologic studies have begun to provide some insight into the heterogeneous character of the disease. These studies clearly separate the genetics of the insulin-dependent from the non-insulin-dependent (adult-onset) form of diabetes. Studies with monozygotic twins reveal a striking difference in the concordance rates: for pairs of identical twins with non-insulin-dependent diabetes, concordance approaches 100 percent; while in insulin-dependent diabetes, it is only 50 percent. As noted in last year's Annual Report, some - though not all - studies on the population association between the HLA system (a system of inherited tissue-type markers found in white blood cells) and diabetes have shown that certain HLA alleles (genes which determine alternative characters in inheritance) appear with greater frequency in insulin-dependent diabetics than in the general population. The genetic factors associated with non-insulin dependent diabetes appear, however, to be different as evidenced by the fact that no such association between HLA type and this form of the diseases has been observed. Studies in this area are continuing.

With respect to a viral etiology of insulin-dependent diabetes, it has been established that viral infections in experimental animals with certain genetic backgrounds may result in extensive pancreatic islet cell damage causing insulin-dependent diabetes. Several types of encephalomyocarditis virus have been shown to infect and destroy the pancreatic islets in certain strains of mice; other strains, however, are resistant to this viral infection. In addition, tissue culture techniques have demonstrated that the genetic constitution of the islet cell determines whether the virus can or cannot attach to the cell surface, penetrate the cell and cause damage.

There is also evidence that viruses may play a role in some forms of insulin-dependent diabetes in man: a suggestive seasonal variation, development of diabetes in close association with some viral infections such as mumps and rubella, and high titers of circulating antibodies to certain viruses. In addition, human viruses have been shown to be capable of inducing diabetes in animal models and damaging cultured animal beta cells: variants of two widely disseminated human viruses, Coxsackie B4 and Reovirus type 3, produced a diabetes-like syndrome in exposed and infected mice. Similarly, scientists at the National Institute of Dental Research have reported that two other human viruses, mumps and Coxsackie B3, were able to infect and damage human pancreatic beta cells in culture. Very recently, this group has also established that Coxsackie B3 virus precipitated

the onset of insulin-dependent diabetes in a 10 year-old suburban Maryland boy. Although this work was not directly supported by the NIAMDD, it is noted here because of its potential significance to research in the field.

Evidence for an immunologic component in beta cell injury comes from the observation that most patients with insulin-dependent diabetes have circulating islet cell specific antibodies (ICA), as well as from the above mentioned association between HLA type and the disorder. This area of investigation is perhaps one of the best examples of the broad implications of sophisticated diabetes-related basic research. The initial promising results of studies on interrelationships of the genetic constitution, immunologic status and susceptibility to viral infections in the insulin-dependent form of the disease are receiving increased research attention. Data from the study of the BB Wistar rat suggest an immunological intervention in the treatment of the disease is being pursued actively.

Diabetes Centers Program

This program provides support for both Diabetes-Endocrinology Research Centers (DERC) and Diabetes Research and Training Centers (DRTC). The primary requirement for establishment of either a DERC or a DRTC is a strong base of high-quality, ongoing biomedical research. In addition, DRTCs also have a training/information transfer component which includes training of both medical and allied health professionals, continuing education, model demonstration and outreach activities. For both types of centers, the grant provides for core facilities (shared resources), pilot and feasibility studies (new initiatives), and limited funds for program enrichment. This funding is intended to strengthen existing programs, increase productivity and foster the generation of new ideas and innovative research approaches.

While the emphasis in the DERC centers primarily around biomedical research, the fundamental objective of the DRTC is broader, and includes the creation of an environment which fosters and integrates the efforts of basic and clinical scientists, physicians, allied health personnel and educators joined together in a cooperative, multi-disciplinary approach to the many research and health care problems associated with diabetes and its complications. Therefore, the DRTC program as a whole, represents not only a concerted effort to achieve a better understanding of diabetes through biomedical research, but also to improve the delivery of health care to the diabetic patient. Although each center is expected to develop its own independent program in accordance with local needs, interests and resources, each is also expected to be responsive to national needs, and must be willing to work cooperatively with the NIAMDD and, in the case of the DRTC, with other federally-supported diabetes programs.

During the past year, the program provided support for 3 DERCs and 8 DRTCs as follows:

Diabetes Endocrinology Research Centers

University of Pennsylvania (Philadelphia)
University of Washington (Seattle)
University of Iowa (Iowa City)

Diabetes Research and Training Centers

Albert Einstein College of Medicine (Bronx)
Indiana University (Indianapolis)
University of Michigan (Ann Arbor)
Washington University* (St. Louis)
University of Chicago* (Chicago)
Joslin Diabetes Foundation, Inc. (Boston)
Vanderbilt University* (Nashville)
University of Virginia* (Charlottesville)

*Previously supported as a DERC.

Both types of centers are being continually assessed and monitored in a variety of ways including annual staff review of comprehensive progress reports and annual program staff visits to each center. An annual center meeting represents yet another mechanism for exchange of information and evaluation of progress. The first such meeting was held last year with center principal investigators and other key participants, representatives from other federal agency diabetes programs which interface with DRTC activities, and NIAMDD staff. The annual meeting, which includes both formal reports and workshops, is intended to address broad issues which have a common overall interest to the centers program. The workshops provide a necessary forum for frank discussion of specific problems, needs and areas of concern.

Inasmuch as activities in the DRTC training/information transfer component represent somewhat new and innovative concepts, more formalized communications (Newsletters, etc.) have been initiated to insure optimal program development. In addition, center participants who are involved in this component have initiated a series of special meetings to become better acquainted with other DRTC training/information transfer programs and to focus attention on areas where cooperation will both prevent duplication and advance progress. Two such meetings have already been held over the past year and a half and have led to a significant exchange of materials and methodologies among the DRTCs. Another meeting is planned for December of 1979. Additional center activities deserving mention include the participation of each DRTC in the recent National Conference on Behavioral and Psychosocial Issues in Diabetes, and a 2 day symposium sponsored by the University of Indiana DRTC on compliance research in diabetes.

The DRTCs cooperate and maintain liaison with two other groups located in NIAMDD - the National Diabetes Information Clearinghouse and the National Diabetes Data Group - as well as with diabetes programs of other federal agencies including the Center for Disease Control and the Indian Health Service. Liaison with both the National Diabetes Advisory Board and the Diabetes Mellitus Coordinating Committee provides additional opportunities to extend this type of interaction and cooperation to other federally funded diabetes programs in the future.

One problem faced by the DRTCs is the difficulty of finding long-term support for research projects dealing with training/information transfer activities. The National Diabetes Advisory Board formally recognized this difficulty and recommended that DRTC support should be expanded to include research studies in these areas. In response to this recommendation, a careful study was made by staff and their consultants. As a result, the DRTC guidelines are now being revised to include limited support of research projects in the DRTC training/information transfer component for periods of up to five years duration.

It has been noted that applications from established DERCs have been significantly more successful in receiving funding for establishment of a DRTC than those from other institutions. This observation has led to the development of a new mechanism of support which will provide an opportunity for institutions to phase the establishment of DRTCs through prior establishment of DERCs. Once established, a DERC will have the opportunity to apply for modest funding in the form of an exploratory grant to search out and develop the resources necessary for initiation of DRTC training/information transfer activities.

In summary, it is felt that both types of centers have progressed during the past year in terms of fulfilling programmatic objectives and goals. It is anticipated that the program will continue to develop and evolve during the coming year.

Clinical Applications Program

The first major initiative of this new program will be to further explore the feasibility of conducting a multi-center collaborative clinical trial to assess the effect of strict blood glucose control on the development of the vascular complications of diabetes. Initiation of such a trial was formally recommended in the Report of the National Commission on Diabetes to the Congress of the United States. More specifically the Commission recommended that "the National Heart, Lung and Blood Institute and the National Institute of Arthritis, Metabolism and Digestive Diseases initiate and support a five-year clinical study to assess the effect of treatment of juvenile-onset diabetes on the development of microvascular and macrovascular complications." Subsequently, an ad hoc advisory committee appointed by NIAMDD and NHLBI considered the general feasibility of such a study, and recommended that a limited study first be conducted to determine the feasibility of establishing and maintaining a "strict control group"

of insulin-dependent diabetic patients with clinically and statistically significant differences in blood glucose control from a more customary or "established treatment group." If this proves feasible, they recommended proceeding with a full scale clinical trial as outlined by the Commission.

Based upon these recommendations, the program recently has developed Requests for Proposals soliciting clinical treatment centers and a data coordinating center to plan (Phase I) and conduct (Phase II) a limited feasibility study. More specifically, the Phase I activities will involve development of a study protocol, necessary forms and Manual of Operations for Phase II by key personnel from the Clinical Centers, the Data Coordinating Center, and the Institute staff, as well as consultants in the areas of biostatistics and epidemiology. During Phase II, the feasibility of establishing and maintaining a high degree of control of blood glucose in an intensive therapy group will be evaluated. Subsequently, if it is determined by an independent Advisory Group that the degree of blood glucose control in the intensive therapy group is clinically and statistically significantly different from that achieved in a group receiving more customary or established treatment, the program anticipates initiating a subsequent procurement for implementation of a full scale clinical trial to test (Phase III) and analyze (Phase IV) the effects of strict blood glucose control on the development of diabetic complications.

If initiated, Phase III will involve random assignment of insulin-dependent diabetics into the two treatment groups (intensive therapy and customary treatment) with periodic evaluation of certain microvascular, macrovascular, and biomedical parameters; as currently envisioned, development of retinal vascular changes characteristic of diabetic retinopathy will be the primary endpoint of the study. During Phase IV, the Data Coordinating Center will complete all analyse from the Clinical trial, will submit final reports to the Institute, and will collaborate with the investigators and Program staff in the preparation of reports for publication.

It should be emphasized that the specific methodologies and protocols for this study will be developed by the participating investigators in order to ensure application of the most recent scientific and clinical techniques. In addition, the results of each phase will be reviewed by an independent Advisory Group prior to initiation of the next subsequent phase of the study. It is anticipated that such an approach will help ensure that this most critical question in diabetes research, namely the relationship between control and complications, is addressed in a fashion which reflects the best collective judgement in biomedical science.

National Diabetes Data Group

The Data Group serves to foster, coordinate and integrate national resources for the collection and analysis of data on diabetes to allow a more rational basis on which to develop scientific priorities and plan public health programs. The Data Group also continues to provide the federal focus to which both the lay and scientific communities turn for accurate, reliable statistics on diabetes.

The National Diabetes Data Group consists of a nationwide group of expert advisors including epidemiologists, individuals with expertise in the research, nutritional and socio-economic aspects of diabetes, as well as representatives from Federal agencies (the National Institutes of Health, the Center for Disease Control, the National Center for Health Statistics, and the National Diabetes Advisory Board) and the private sector (the American Diabetes Association and the Juvenile Diabetes Foundation).

A major probelm in assessment of the scope and impact of diabetes is the lack of a satisfactory diagnostic and classification scheme for the various forms of the disease. To address this issue, the Data Group sponsored an international work group which formulated a consensus on a uniform classification of diabetes, appropriate diagnostic criteria, and a standard terminology to be used as a guide for clinical and epidemiologic research. During the past year, these proposals were widely circulated within the U.S. and similar versions were reviewed abroad in an effort to obtain an international consensus. Recently, these proposals were presented to the Board of Director of the American Diabetes Association and it is anticipated that they will be considered for endorsement by the International Diabetes Foundation.

In an effort to further stimulate interest in the epidemiology of diabetes, the Data Group and the National Institute of Child Health and Human Development cosponsored a symposium on "Childhood Diabetes and Diabetes in Pregnancy" at the annual convention of the American Public Health Association. Further, the Data Group supported an analysis of the epidemiologic literature on obesity as a cause of diabetes, in order to assess research needs in this aspect of the etiology of adult-onset diabetes. In collaboration with the Statistical Bureau of the Metropolitan Life Insurance Company, the Data Group recently estimated that the economic costs to the Nation due to diabetes in 1977 approximated $6.8 billion. This estimate includes the direct and indirect costs of medical care of diabetics and earnings loss due to disability and death, but excludes costs associated with the complications of the diseases.

During the past year, the Data Group also has been working closely with officials from the Health Interview Survey, National Center for Health Statistics, to analyze the surveys that have been collected to date and prepare this data for publication in the medical literature. The information from these data sets should provide a more complete and accurate analysis of the Epidemiology of diabetes than has been available in the past. The Data Group has also arranged for early analysis of the data on diabetes now being collected through the Health and Nutrition Examination Survey, National Center for Health Statistics. In addition, from data reported in the 1976 Health Interview Survey, the Data Group analyzed aspects of vision impairment for the Center for Disease Control in order to help establish priorities for diabetes screening programs.

Publication:

Harris, M.I. and Siebert, C.W.: Analysis of Demand and Supply Data. In A Study of Insulin Supply and Demand. DHEW Pub. No. (NIH) 78-1588. Washington, D.C., U.S. Government Printing Office, 1978, 27 pp.

National Diabetes Information Clearinghouse

The National Diabetes Information Clearinghouse was established by the Institute in Fiscal Year 1978. It serves as the central resource for the collection and dissemination of information about educational and scientific materials, programs, and other resources relevant to diabetes. The Clearinghouse serves and interacts with the Diabetes Research and Training Centers, local, regional, and national diabetes organizations, professional groups, state departments of health, and other federal agencies.

The Clearinghouse, in its initial period of development, has three major goals: to increase the availability of accurate diabetes information for health care professionals, for patients, and for their families; to increase community awareness and understanding of diabetes as a major health problem; and to establish a viable system for speedy communications about new developments, techniques, and programs in diabetes among all sections of the diabetes community. In cooperation with the Clearinghouse, the American Diabetes Association, Juvenile Diabetes Foundation, and American Association of Diabetes Educators - the three major national diabetes organizations - are establishing criteria and a mechanism for evaluating non-scientific print diabetes education materials. These groups are also cooperating with the Clearinghouse to further define the educational and informational needs of the diabetes community.

During the past year, the Clearinghouse has compiled eleven annotated bibliographies in response to requests for information from health care providers, diabetics, and the public. These bibliographies, which range from 30 to 130 items, include topics such as Care of the Diabetic Foot, Educational Materials For and About Young People with Diabetes, Cookbooks for Diabetics, and Spanish Language Materials for Diabetics. At the request of the National Diabetes Advisory Board, the Clearinghouse also has provided the Emergency Medical Service, Department of Transportation, with diabetic emergency materials appropriate to first responders. In addition, the Clearinghouse has developed a Thesaurus of Diabetes Terms for libraries and other reference sources to facilitate access to information related to diabetes materials. In response to a joint request from the American Diabetes Association and the Juvenile Diabetes Foundation, Clearinghouse staff are compiling a Handbook of Diabetes Terms modeled after the highly useful Handbook of Heart Terms. In addition, at the request of the Indian Health Service, the Clearinghouse is coordinating development and production of a simple diet manual of ethnic Indian foods in a format that is comprehensible to patients with low reading abilities.

The members of the Clearinghouse Advisory Committee represent the three national diabetes organizations, the Diabetes Research and Training Centers, the Center for Disease Control, State departments of health, and the

National Library of Medicine. The expertise of this group spans diabetes-related nursing, diet and nutrition, clinical medicine and education, and includes consumers and representatives from other established clearinghouses.

Publications:

Lipsett, L.F.: Birth of a Clearinghouse. Diabetes Care 1: 308-309, 1978.

Lipsett, L.F.: Bibliography: Diabetes-Related Psychosocial and Behavioral Studies. The Diabetes Education 1: 8-11, 1979.

Salans, L.B. and Lipsett, L.F.: Juvenile Diabetes: The Forever Disease. Pharmacy Times 45: 38-42, 1979.

ENDOCRINOLOGY, METABOLIC DISEASES AND RESOURCES PROGRAMS BRANCH

During FY 79 this branch was established to coordinate the endocrinology, metabolic diseases, manpower, and resources development activities of the cluster. In addition, the office serves as a focus for administration of the NIAMDD cystic fibrosis-related activities.

A major effort has been directed by the Institute during FY 79 toward increasing investigators' awareness of the needs and opportunities in CF research. This initiative resulted from a series of recommendations in the report entitled "Cystic Fibrosis: State of the Art and Directions for Future Research Efforts" that was completed for the NIH by the Cystic Fibrosis Foundation during FY 78.

Two publications in a series entitled "Cystic Fibrosis: A Disease in Search of Ideas," one on basic sciences and the other on clinical sciences, were prepared and distributed to the scientific community. A third volume on model systems has been prepared and will be distributed to veterinarians and comparative pathologists who might develop new model systems, and to cell biologists, physiologists, and other individuals who might be interested in utilizing existing model systems to study CF. The NIAMDD also participated in an NIH-wide program announcement informing investigators of our interest in supporting high quality research in CF.

In addition to these publications, the Institute co-sponsored with the Cystic Fibrosis Foundation a working conference on cystic fibrosis. Nearly 100 investigators were invited to attend the conference. The purpose of the conference was to bring together scientists representing many disciplines, including cystic fibrosis researchers as well as researchers who have not previously studied cystic fibrosis. The workshop attempted to refine the recommendations of the "State of the Art" report and research directions that will give the promising short-term and long-term research answers to the problems of cystic fibrosis. In addition, it is hoped that the conference served as an additional mechanism to attract scientists into cystic fibrosis research.

During FY 79 the Institute received 52 applications in CF-related research. This compares with 17 and 25 applications received during FY 78 and 79, respectively. The awarding of a number of meritorious programs will allow for a 50% increase in the Institute's commitment to support of CF-related research.

New information is already beginning to result from these new programs, especially as they relate to the development of procedures for detecting asymptomatic carriers of the CF gene (heterozygotes) and for performing prenatal diagnosis of the disease. Last year's annual report discussed the potential diagnostic value of the dexamethasone sensitivity of fibroblasts for CF patients. This year, NIAMDD-supported investigators are exploring several additional methods for developing new diagnostic and detection procedures for the identification of the CF gene.

Several years ago, NIAMDD-supported investigators described abnormalities in the alpha-2-macroglobulin-protease complex in patients with CF. It has been postulated that the deficiency of arginase esterase activities associated with this complex could account for the elevation of the levels of certain polypeptides or protein factors thought to be of possible etiologic importance in CF. The measurement of the levels of the protease activity was initially difficult in that the procedure required a tedious enzymatic assay. However, these same investigators have recently identified a chemical, 4-methylumbelliferylguanindino-benzoate (4MUGB), that can be used to quantitate the levels of the proteolytic enzymes in serum and tissue specimens.

Initial studies using MUGB have demonstrated quantitative differences in the amount of protease activity between cultured fibroblasts obtained from control subjects, cystic fibrosis patients, and asymptomatic heterozygote carriers. In addition, these studies were recently extended to human amniotic fluid obtained by amniocentesis. Measurement of protease activity obtained from three women who subsequently delivered children with CF has shown that MUGB-reactive protease method may be a useful marker for the prenatal detection of CF.

Like the other diagnostic procedures currently being developed, further refinements are needed in the assay procedure in terms of assuring its reproducibility and reliability before it can be used for large-scale screening for the CF gene or trait.

Highlights of the branch's programs are summarized as follows.

Endocrinology Research Program

A major emphasis of the Endocrinology Research Program is to support studies directed at determining how hormones act at the cellular and subcellular level in the organism. This program supports a number of studies that are attempting to determine the relationship of hormonal structure to function and the role played by cellular and subcellular membranes in hormone action. Other areas of emphasis include studies on secretion mechanisms of hormones, hormonal precursor molecules, and the synthetic processes used by the cell to produce hormones. In addition to these basic studies, the program also supports a number of clinically related studies on the pituitary, thyroid, parathyroid, and adrenal gland hormones. Studies on more recently discovered hormones, e.g., peptides from the hypothalamus such as endorphins and encephalins and on hormone-like substances, such as prostaglandins, are also supported.

Highlights from some of the work supported by the Endocrinology Research Program follow.

Hormone Production

Currently, many of the hormones used for treatment of endocrinopathies must be extracted from glands of humans and animals. The methods for

extracting the hormones are frequently tedious and expensive and, in many cases, the availability of the glands is decreasing. A significant step in overcoming this problem was recently reported by NIAMDD-supported investigators. These investigators have utilized recombinant DNA techniques to produce rat growth hormones in bacteria. This is a significant step in the application of recombinant DNA, and it suggests that with further refinements, the technique can be used to produce human hormones, such as growth hormone and insulin, which are currently in short supply.

Growth Factors

In addition to growth hormone, the Institute is supporting projects that are attempting to identify and characterize other growth factors. One factor has been isolated from the Plerocercoid larvae of the tapeworm, *Spirometra mansonoides*. Institute-supported investigators are attempting to establish the biochemical and physiological relationship of this growth factor to mammalian growth hormone and other growth factors such as insulin and somatomedin. Plerocercoid growth factors (PGF) have been shown to produce responses in mammals similar to those attributed to growth hormone. In a series of experiments that were conducted to determine the effects of PGF on the onset of puberty in rats, it was demonstrated that PGF delays puberty while allowing normal growth rates. If growth hormone treatment is administered in conjunction with PGF treatment, puberty is restored to normal. It is possible to conclude from this work that growth hormone has a direct effect on puberty onset exclusive of its effect on growth. This investigation has further shown that a reduction of growth hormone levels in PGF-treated rats delays puberty.

Diabetes

Reproductive dysfunction is common in human diabetics and experimental animals. Several studies are supported which are attempting to characterize various hormonal deficits found in diabetics and to find some molecular bases of reproductive failure in individuals with this disorder. It has been found, for example, that the binding sites for both testosterone and estrogen appear to be decreased in diabetic animals. These data suggest that one of the effects of diabetes may be to reduce these hormonal binding sites, which may lead to a decrease in sexual function.

Releasing Factors

By using micromethods, Institute-supported investigators are attempting to understand the interrelationships between hypothalamus and pituitary in controlling the release of pituitary hormones. In one study, luteinizing hormone releasing hormone (LHRH) was detected in the hypophysial portal plasma following electrical stimulation of the medial preoptic area of the brain. This observation suggests that this may be an important controlling point in the subsequent release of luteinizing hormone (LH) from the pituitary. These same investigators have also shown that

various prostaglandins stimulate release of LH from the pituitary by enhancing release of LHRH from the hypothalamus. Using these same delicate techniques, the investigators have also been able to detect the presence of pituitary hormones in the portal system that return blood toward the hypothalamus. The data, using the microcannula techniques, are already providing investigators with new insights about the mechanism by which the nervous sytem, the circulatory system, and the endocrine system interact in controlling bodily functions.

Hypothyroidism and hypogonadism are frequently observed in comatose patients after several weeks of hospitalization. Institute-supported investigators have observed that there is a loss of production of both luteinizing hormone releasing hormone (LHRH) and thyroptropin releasing hormone (TRH). The reduction of these releasing factors may account for the corresponding decrease in the TSH and LH levels, which in turn contribute to the hormonal dysfunction associated with comatose individuals.

Studies of releasing factors from the brain's hypothalamus have been enhanced by using analogs of these fairly simple molecules which prolong certain of the physiological activities exhibited by the natural hormones. A superactive analog to LH-RH has been developed which has antifertility effects in female rats. The investigator has also shown that the analog has extra-pituitary effects by decreasing the number of uterine binding sites for estradial in rats. The analog given in large doses blocks implantation and terminates gestation when given post-coitally to rats. The effect appears to be directly related to the hypersecretion of LH, functional luteolysis, and/or inhibition of progesterone secretion.

Thyroid Hormone

The thyroid gland synthesizes and releases the thyroid hormones in two major forms, T3 and T4. These two hormones are responsible for influencing many metabolic processes, and, in addition, it is believed that they regulate their own production by inhibiting production of thyroid stimulating hormone (TSH) at the level of the pituitary.

Using tissue culture techniques, a group of Institute-supported investigators have presented data which suggest that T3 is approximately 15 times more potent than T4 in regulating TSH production and cellular metabolism. It appears that these effects may be initiated by interaction with nuclear binding sites. Their data also have shown that within the physiological range, low levels of thyroid hormones stimulate and higher levels inhibit TSH production.

Another group of investigators have been examining the control of thyroid hormone production by infusing thyroid stimulating hormone (TSH), thyroid releasing hormone (TRH), and T3 and T4 into non-anesthetized intact rats. It is generally agreed that T3 is the active form of thyroid hormone. In order to study whether or not T4 itself causes an inhibition of thyroid stimulating hormone secretion, this group of investigators has infused both T4 and T3 in separate experiments. They conclude that T4

itself is able to exert some feedback inhibition of TSH secretion independent of its peripheral conversion to T3. Another interesting aspect of the feedback regulation of TSH secretion is to determine whether the feedback information received from a given amount of hormone per 24-hour period is different when delivered continuously than when it is presented in a pulsatile manner. The total daily production of T3 in the rat was calculated. This amount of T3 was infused at a constant rate over a 24-hour period in one group of rats. In another group of rats, the same amount of hormone was given in two bolus injections in a 24-hour period. The results demonstrate that the pulsatile administration of T3 provides greater feedback inhibition of TSH secretion than does its continuous infusion.

National Pituitary Agency

An important activity of the Endocrinology Research Program is the support of the National Pituitary Agency (NPA). Since 1963, this agency has been responsible for both the extraction of human growth hormone (hGH) from human cadaver pituitary glands and distribution of the hormone to clinical research centers around the country.

Clinical research conducted on hGH at these centers has increased the understanding of human growth hormone. In addition, children with short stature involved in the research protocols have benefited from the hormone that is now available to permit them to grow to an essentially normal height.

Other human hormones including human follicle stimulation hormone, luteinizing hormone, and prolactin have also been extracted and distributed by the NPA. It is expected that clinical studies will be initiated on these hormones in the near future. In addition, other hormones from rat and sheep, distributed by the NPA, are used extensively by the research community. The NPA also produces and distributes kits for radioimmunoassays, a sensitive method for determining amounts of hormones in body fluids.

Metabolic Diseases Research Program

The Metabolic Diseases Research Program provides support for investigator-initiated research into enzymatic mechanisms and regulation, biological transport, and membrane structure as they relate to understanding the etiology and pathogenesis of inborn or acquired errors of metabolism.

Inborn errors of metabolism refer to a group of genetic disorders resulting from an absence or, in some cases, alterations in the activity or structure of enzymes governing metabolic steps essential to the normal performance of body functions. Cystic fibrosis, fucosidosis, Fabry's and Gaucher's diseases are some examples belonging to this category. These diseases can lead to serious, catastrophic illness that can result in a vast spectrum of clinical manifestations, including severe physical deformities, mental retardation, and premature death.

Acquired errors of metabolism refer to alteration in normal enzyme activities triggered by regulatory hormones like insulin, glucagon, and others, lack of vitamins essential for normal enzyme function, microbial or viral infections, etc. Diabetes mellitus is a typical representative of such disorders.

A summary of some significant achievements of the program for FY 79 are presented below.

Inborn Errors of Metabolism

Fabry's Disease (or α-Galactosidase A Deficiency)

This condition is a genetically transmitted inborn error of sugarbound fat (glycolipid) metabolism. The glycolipid, called globoside, contains a sugar chain with a galactose end unit which, under normal conditions, is hydrolyzed by the enzyme α-galactosidase A (α-GA). Complete or partial absence of active α-GA results in accumulation of globoside in various tissues, particularly the cardiovascular-renal system. Clinical manifestations of Fabry's disease include the appearance of numerous purple skin lesions, corneal opacity, and episodic crises of excruciating pain at extremities. Death usually occurs during the fourth or fifth decade of life due to progressive renal failure. Recently a group of Institute-supported investigators have undertaken enzyme replacement therapy in Fabry's disease with partially purified human spleen and plasma α-GA forms (isozymes). They infused two afflicted brothers six consecutive times over a 117 day period. Although both isozymes reduced circulating globoside levels by half, significant differences in their physiologic behaviors were observed. The splenic isozyme had very short circulating half-life and allowed a return to preinfusion concentration of globoside within two to three hours. In contrast, the infused plasma isozyme had a relatively long circulating half-life and allowed a gradual return to preinfusion levels in 36 to 48 hours. Thus, the plasma isozyme cleared about 25 times more globoside over time than the splenic isozyme. A second dose of plasma isozyme, 48 hours after the initial dose, further decreased the glycolipid level to the normal range. Since no adverse immune response to the infused isozymes was detected by skin or immunodiffusion tests, and the injections were well tolerated, these results indicate the feasibility of enzyme therapy in Fabry's disease with the appropriate α-galactosidase A isozyme.

Fucosidosis

This condition is a rare genetically transmitted neural storage disease occurring in children. Biochemically, the disease involves a deficiency of α-L-fucosidase activity coupled with tissue accumulation of fucose-containing sugars and polar glycolipids. Progressive cerebral degeneration, muscle weakness and spasticity, thickening of the skin and respiratory difficulties result in death usually during the first decade of life.

An Institute-supported researcher performed extensive biochemical studies on the liver and spleen of a five year old deceased patient. The patient's liver was found to retain 25-50% of normal α-L-fucosidase activity. A similarity in thermostability, kinetic, and immunochemical properties was observed between the patient's crude enzyme and crude enzyme from a normal liver. Following partial purification, the patient's liver α-L-fucosidase was found to possess good activity. However, a fraction of the patient's liver not containing the enzyme was found to significantly inhibit normal liver α-L-fucosidase activity. The fact that, to date, no evidence is available for a structurally altered α-L-fucosidase in fucosidosis, coupled with the first demonstration of an α-L-fucosidase inhibitor, opens the way toward elucidation of the biochemical defect in this disease.

Purine Related Immunodeficiency Disease

Lymphocytes are cells which produce serum antibodies able to attack foreign materials. Lymphocytes can be subdivided into at least two classes: B-cells produced by the bone marrow, responsible for the synthesis and secretion of antibody molecules; and T-cells, produced by the thymus, responsible for helping the B-cells perform their task, as well as destroying foreign tissues. T-cell and B-cell dysfunction can result in various immunodeficiency diseases.

It was recently shown that two enzymes catalyzing sequential reactions in the purine degradation pathway, namely, adenosine deasminase (ADA) and purine nucleoside phosphorylase (PNP), are associated with clinical immunodeficiency states. The former causes an accumulation of deoxy-adenosine triphosphate in erythrocytes of ADA-deficient children, resulting in severe T-cell and variable B-cell dysfunction. The latter causes elevated levels of deoxyinosine in urine of patients with PNP deficiency and only disturbs T-cell function.

An Institute-supported research team has established that cancerous lymphoblast lines of T-cell origin are selectively killed by low concentration of deoxyadenosine and are more sensitive than B lymphoblasts to deoxyguanosine. Also, extremely low concentrations of deoxyadenosine were toxic to one kind of mouse leukemia cells. The drug dipyriadamole was found to inhibit toxicity of the accumulating deoxyadenosine. New and exciting research areas are thus opened by demonstration of: (a) possible anticancer activity of deoxyadenosine; or (b) potential therapeutic use of dipyridamole in correcting ADA and PNP deficiencies.

Acquired Errors of Metabolism

Diabetes Related Research

The increase in total body glucose in diabetes is due to reduction in rate of glucose conversion to carbon dioxide and thus to a disorder in carbohydrate metabolism. Quantitative information on activity-concentration relationship of intracellular tissue enzymes involved

in the metabolic processes could shed light on the nature of differences between normal and diabetic states. Assaying for enzyme activity in tissues might not provide an accurate picture of total enzyme concentration since inactive forms of the enzyme could be present in addition to the active form. Furthermore, there could be variations in enzyme activity or concentration due to regulatory hormones, diet, and disease.

An Institute-supported grantee has developed radioimmunoassays for quantitating tissue concentrations of two enzymes involved in carbohydrate metabolism: fructose-1,6-diphosphatase (FDP) and pyruvate kinase (PK). The radioimmunoassays which utilize antibodies grown to the pure enzymes were used in combination with methods measuring enzyme activity in tissues.

Alloxan-induced diabetic state produced significant increases in both the concentration and assayable activity of rabbit liver FDP. In all cases, the severity of the induced diabetes paralleled the increase in enzyme concentration and activity. Since blood glucose level is dependent on insulin concentration, the possibility that insulin is a regulator of FDP synthesis was postulated. In the case of pyruvate kinase, different concentration versus activity ratios were obtained for the rabbit muscle and rabbit heart enzyme. Also, alloxan-induced diabetes changed heart PK activity, while it did not affect the activity of muscle PK.

These findings and further studies relating enzyme concentration and activity in tissues have far reaching applicability. Not only could they be extended to other enzymes and diseases, but they could also be utilized to verify the assumption that some aspects of aging could be a result of changes in concentration and activities of various enzymes.

Model System for Enzyme Studies

Translation of Messenger Ribonucleic Acid (mRNA) in Yeast

Yeast is an important model for study of enzymes and mutant enzymes with reduced catalytic activity. Directed and targeted synthesis of a specific protein in yeast or a yeast mutant could provide an understanding of errors in metabolic processes.

An Institute-supported group of researchers elucidated the mechanism of *in vitro* translation of mRNA in cell-free extracts from yeast by analysis of intermediary reaction and isolation of some intermediary complexes involved in the protein synthesis. The cell-free solution contained no mRNA or amino acids; it contained, however, ribosomes, tRNA, and all initiation factors required in the process. After addition of methionine, mRNA, and all other amino acids, the protein synthesis commenced and the nature of mRNA determined the protein synthesized. This represents the first system from a fungi capable of initiating protein synthesis and translating natural mRNA. In yeast, these methods could help to identify the genetic lesions in mutants with altered protein synthesis.

Resources and Manpower Development Program

Manpower Development Program

The continued development of research programs in the areas of diabetes, endocrinology, and metabolic diseases is dependent on an adequate supply of well-trained scientists, and the mission of the Manpower Development Program is to assure that such a supply is continuously available. Toward this end, the Manpower Development Program provides support for a full range of research training and research career development awards.

A particular focus of interest is the development of physician-scientists to carry on clinical research. Medical training and a background in patient care permit a scientist to pose research questions different from those posed by a scientist exclusively trained in a laboratory. Physician-scientists play a critical role in the translation of laboratory and field research to the problems of the patient. The research physician is also able to identify and react immediately to new and unanticipated responses from his research patients and to develop hypotheses that can be incorporated into ongoing research projects. A significant decline in these critically needed investigators has occurred in the past decade. This decline has been documented in several recent studies, including the 1978 report of the National Research Council entitled Personnel Needs and Training for Biomedical and Behavioral Research. Responding to this problem, the DEMD cluster has given emphasis to awards targeted on physicians preparing for careers in academic medicine and research.

Training

All training supported by the Institute is under the authority of the National Research Service Act. Both individual and institutional awards are available for training at the postdoctoral level, and a few institutional training programs provide predoctoral support in selected areas.

Over the past several years, DEMD cluster staff have devoted special effort to expanding the training program, particularly in diabetes and related areas. As a result, several dozen new institutional training programs have been initiated, and, by 1978, over 175 individuals were being supported through individual fellowships. In keeping with the concerns for developing physician-scientists, emphasis has been given to research training programs for physicians. Institutional training programs prove to be a very important mechanism in that they allow the stable development of a coordinated training effort at a given institution over a period of at least five years.

Although there has recently been a dramatic expansion of the extramural training effort in DEMD, the 1979 training budget was exactly equal to the amount spent for training in 1978. Thus, the program was limited to an essentially steady state condition. Even so, four new training programs were begun, and over 50 new individual fellowships awarded.

A particularly difficult problem for all awardees--both institutional and individual--concerned the stipend levels for trainees, which had not been raised since the National Research Service Award program was initiated in 1975. Training program directors indicated that until such adjustments are made, recruiting trainees will continue to be difficult, particularly for training programs involving physicians.

Career Development

Career development awards provide salary support, and, in some cases, limited support for research activities to individuals who have completed their formal training but who have not yet reached the status of independent, established investigators. Two of these awards, the Clinical Investigator Award and the Research Career Development Award, are available in all fields of investigation. The former is designed for physicians or other clinically trained individuals to develop their research skills by providing three years of salary support and a limited budget for research expenses. The latter is a five-year salary award to assist an investigator to become "established" as a productive scientist in his field.

The Special Emphasis Research Career Award was developed during 1978 and 1979 as a response to critical shortages of physician-scientists in particular areas related to diabetes. The NIAMDD developed this award in cooperation with the NHLBI and the NICHD. One version of the award is targeted on cardiovascular, endocrinologic, and metabolic aspects of diabetes, the other on obstetrical, neonatal, and pediatric aspects. The awards offer physicians and other clinically trained persons five years of salary support and a limited budget for research expenses. Awardees pursue a structured program of their own design, under the sponsorship of one or more senior investigators in the field, aimed at developing those research skills which are required to attack critical research questions posed by diabetics but which transcend traditional disciplinary boundaries. The first awards in the cardiovascular area were made in late FY 78, and the first awards in the obstetrical, neonatal, and pediatric areas will be made in FY 80.

New Investigator Award in Diabetes

In order to encourage newly trained investigators to enter research in diabetes, the Institute initiated this non-renewable, three-year award in 1979. It is open only to scientists who have not yet served as principal investigators on regular research grants funded by the Public Health Service. Maximum allowable direct costs budgets are $90,000 for three years and $35,000 for any one year. The first round of competition brought 36 applications of which 32 were approved.

Resources Program

The DEMD cluster is developing a program to supply some special research resources to our grantees, especially in areas related to diabetes, but potentially in other areas as well. First priority is given to substances

or animal models which are necessary to pursue important lines of research but which are not presently available from commercial sources.

As a result of an RFP issued last year, we are in the process of supporting, through contracts, the preparation of guinea pig and chicken insulin and desoctapeptide insulin. It is our hope to be able to respond to any other defined needs in this area by initiating the supply of other such materials for the study of insulin structure/function relationships.

Animal models are extremely important as tools for research in diabetes, endocrinology, and metabolic diseases. It is not necessary that any given model duplicate every aspect of a particular human disease, but rather that the primary disturbance be demonstrated in an animal where genetic modifiers can be more easily defined than in man. The Institute has been active in soliciting research in the area of the characterization of such models relevant to diabetes. We are now supporting such work in at least twelve different animals with many studies being done in the ob/ob and db/db mice, the Chinese hamster, and, more recently, in the BB Wistar rat.

ARTHRITIS, BONE, AND SKIN DISEASES PROGRAM
ANNUAL REPORT - 1979

ARTHRITIS RESEARCH AND TRAINING PROGRAMS

The major emphasis in the Arthritis Program is with individual investigator initiated research grants concerned with the following: (1) general approaches to rheumatic disease including etiology, metabolism, immune disturbances, antibody and autoimmune disease and rheumatoid factor; (2) research differentiated to categorical disease such as rheumatoid arthritis, degenerative joint disease, systemic lupus erythematosus, heritable disorders, etc.; (3) undifferentiated research related to articular cartilage, collagen, connective tissue, basement membrane and synovial membrane and fluid; and (4) related musculoskeletal studies involving the structure, function, contraction and metabolism of muscle. The Arthritis Program budget is divided approximately equally over these four major areas. The total research budget in FY 1979 was $17.6 million, in support of 181 projects. Training for the same period was $1.27 million to support eleven individual fellowships and twenty institutional fellowship awards. Support for the research career program expanded to $1.06 million to include twenty Research Career Development Awards and eight Clinical Investigator Awards.

Etiology of Arthritis. Several model systems have shown that viruses can induce or enhance auto-immune manifestations with concomitant auto-immune disease. These data, coupled with scattered reports of the induction of rheumatoid factor following several virus infections, indicate heightened antibody responses to several viruses in patients with connective tissue disorders. Other data have continued to stimulate and direct interest in the search for infectious etiologies. The use of organ cultures, functional synovial cells, together with the use of various mitogens and metabolic inhibitors to enhance test sensitivity, may provide positive information. A novel approach is planned in the use of a temperature sensitive mutant of a virus to detect possible viral-enveloped glycoproteins from persistently infected cells. It is essential to continue this search for virus material in cells; the subject has been widely discussed but still lacks firm support.

One grant of the Program supports a broad based epidemiologic and clinical study of the putative cause of Lyme arthritis, namely the presumed infection of an arbovirus transmitted by ticks. Recent patient populations have extended the geographic distribution well beyond the original community of Lyme, Connecticut. The recent studies show that neurological features and myocardial involvement form part of the natural history of this syndrome. Chronic arthritis is observed in approximately 8% of the patients. This arthritis becomes erosive and destructive. Cryoglobulins present in over half the patients suggests that the disease can be viewed as a systemic immune complex disorder. Lyme arthritis is proposed as the first truly infectious disorder which shows chronic changes typical of rheumatoid arthritis.

Immune Disturbances in Arthritis. The large disturbances in the immune system which occur during arthritis are the fount from which considerable arthritis-related research flows. Some of the research is fundamental, such as studies on the structure of immunoglobulins. Certain diseases involving immunoglobulins are related to mutants affecting the structure of the heavy chain subunits. These mutants cause deletions in the hinge region of the heavy chains and are providing information about the evolution of the hinge region and its genetic control.

Another grantee is looking at a unique form of immunoglobulin-rheumatoid factor complex found originally in three rheumatoid arthritis patients, in order to assess the universality and biological relevance of the complexes. The study will try to determine if this self-association complex is a general phenomenon, if mediators of inflammation are involved, and whether the complex appears in both plasma and synovial compartments. Further work will attempt the difficult characterization of these complexes, an important immunochemical problem.

Rheumatoid Arthritis. There is considerable research into the cellular pathology underlying symptoms of rheumatoid arthritis. Some of the proposed work stems from the hypothesis that the proliferative response of mesenchymal cells in rheumatoid arthritis depends upon collagenolysis, and that collagenases are the rate-limiting enzymes in mammalian collagen destruction. One of the major efforts will be to assess factors controlling synthesis and release of collagenase from fibroblasts in order to probe and suppress the proliferative lesion in rheumatoid arthritis. Culture systems will be used to search further for collagenase-stimulating factors in the circulation, synovial fluid, or synovial tissue of rheumatoid arthritis. An additional project will question whether collagenase and collagen are synthesized by the same cells.

Systemic Lupus Erythematosus. This is a serious, potentially fatal immunologic disease. Studies on human populations have shown improved outlook for both juvenile and adult populations. Understanding the pathological processes involved in the disease should be improved using a newly developed colony of dogs with autoimmune diseases, including SLE. The colony is being developed as a valuable resource. To complement this laboratory colony, field data are now being collected on SLE and SLE-related conditions in a highly inbred population of Shelty show dogs. A high incidence of SLE and other autoimmune diseases has been demonstrated in this highly inbred population of show dogs. Several generations of these dogs with extensive pedigrees are on record and are available for laboratory and clinical workups. The studies with the Shelties will provide much of the ground work for proposed studies to elucidate the genetic aspects of autoimmunity and the devleopment of clinical disease. The dog resources in this program may ultimately prove to be a model to study the pathogenesis of autoimmunity and autoimmune disease in man. Work continues on murine (mouse) models of SLE.

Osteoarthritis. The Arthritis Program supports research into the fundamental physiology and cell biology of osteoarthritis. One grantee is studying how the environment of the chondrocytes (cartilage cells) affects the nature and rate of synthesis and turnover of collagen and glycosaminoglycans. These

studies were stimulated by the following accumulated data: (1) The observation that articular cartilage is formed of type II collagen fiber within a matrix of chondroitin-4 and -6 sulfate. (2) In osteoarthritis, type I collagen as well as type II is found in cartilage. (3) In vitro chondrocytes isolated from rabbit articular cartilage undergo "depression" and synthesize type I, II, and an unidentified collagen type while decreasing markedly the amount of type II collagen synthesized. (4) This change in phenotypic expression can be enhanced by agents such as 5-bromo-2-deoxy uridine and retarded by high density cultures. The studies will examine culture conditions in a systematic fashion to determine why these modulations occur. Methods of chondrocytic isolation and culture and identification of collagen types and subunit peptides have been carefully worked out as well as methods for measuring each glycosaminoglycan in the presence of others.

The role of proteases in the degradation of cartilage in degenerative and rheumatic joint disease is also being investigated. Since the loss of metachromasia in cartilage proteoglycan is the first detectable abnormality in osteoarthritis, it appears likely that proteases are responsible for early cartilage damage. An understanding of the mechanisms of these enzymes is sought through the purification and characterization of neutral metalloproteases from human and animal cartilage; their localization in normal and arthritic cartilage and the development of a direct assay for collagenase determination.

Gout. Disturbances in urate metabolism are a primary cause of gout. The recent discovery of a defective form of an enzyme involved in urate metabolism promises increased understanding. The mutant enzyme is a kinetic variant of phosphoribosylpyrophosphate (PPRP) synthetase which has increased enzymatic activity and overproduces urate. All prior enzyme defects have linked decreased enzyme activity to overproduction of urate. Of the population with (PPRP) synthetase overactivity, mutants of several types have been observed. Some of the mutant enzymes have an increased affinity for ribose-5-phosphate, while others have an increased inherent specific activity. It now seems likely that the specific structural alteration of the mutant enzyme can be identified. In separate studies a pivotal role for glutamate in the reutilization pathway of purine biosynthesis was found and the importance of glutamate-glutamine interconversion was studied. The site of control of glutamate was traced to its effect on the level of PPRP in the cell.

Notable progress in the understanding of chondrocalcinosis was found in the observation of several fold increases in pyrophospahte concentration in the media of fibroblasts and chondrocytes from patients with the disorder. A more sensitive assay for pyrophosphate has been developed and further studies of the control of pyrophosphate metabolism are under way.

Muscle Structure and Function. There are a number of studies associated with the structure and function of muscle: the structure of myofibrils, regulatory proteins, sarcoplasmic reticulum, the structural basis and dynamics of cellular function and movement, and the structural heterogeneity of skeletal muscle fibers. Other studies concerned with muscle contraction involve projects associated with: the contractile mechanism, contractile proteins,

the biology and regulation of contraction and the mechanical control and energetics of contraction. Finally, a small number of projects are associated with the basic biochemistry and metabolism of muscle.

BONE DISEASES RESEARCH AND TRAINING PROGRAMS

The Bone Diseases Program supports basic and clinical studies of the musculoskeletal system in normal and disease states. The total research and training budget increased to approximately $12.4 million for FY79. Additionally, the number of applications has risen to about 185; therefore, a limited number of the most meritorious research projects were funded in FY79.

A comprehensive training program is the lifeblood of a research program. The number of recipients for Research Career Development Awards has increased to 15. The Clinical Investigator Awardees remain constant at three. Currently, 16 individual fellowships and 10 institutional training grants have been awarded. The total training picture is promising.

Two areas have received special program emphasis: Osteoporosis and Osteogenesis Imperfecta. Previous conferences supported by this program have provided research directions in osteoporosis. Two new grants have recently been funded to (1) ascertain the success of preventive measures in osteoporosis and (2) determine the prevalence of osteoporosis secondary to diabetes. In June 1979, a workshop was held at NIH entitled "Directions for Research in Osteogenesis Imperfecta." State of the art research findings and future needs were discussed by leading authorities. Additionally, two new research grants were funded in the Osteogenesis Imperfecta (OI) area: (1) hereditary factors and (2) electric stimulation to enhance bone growth in OI patients.

Two other conferences were also supported in the past year. A group of about 40 researchers and clinicians gathered for three days to evaluate the state of knowledge on electrical stimulation of bone growth. Two major clinical applications on patients with nonunion fractures for an average of two and one half years showed about 70% healing. The basic researchers have been less successful in clearly identifying the mechanisms by which this technique achieves its improved bone growth. A second area of support was for the Gordon Conference on Bone and Teeth. This annual meeting provided a free flow of information dealing with basic bone growth and metabolism problems.

Bone Metabolism. The range of research endeavors supported by the Bone Diseases Program is broad, varying from basic studies on bone metabolism to clinical and applied projects involving bone diseases such as osteoporosis, Paget's disease and Osteogenesis Imperfecta.

More than 20 grantees are supported by the Bone Diseases Program for basic studies on bone cells, bone formation and bone metabolism. Many are looking at bone formation and resorption through hormonal and other influences. A grantee from Yale University is attempting to determine by immunological means the origin of bone cells in bone remodelling. Another from the University of Connecticut is doing studies revolving around the monocyte-macrophage system in bone resorption. Two grantees are looking at structural relationships in cells by examining the interaction of ground substances in cortical bone and delving into the interactions of mineral constituents.

Other grantees are looking at the processes under which mineralization takes place. A grantee from the University of South Carolina has provided evidence that the matrix vesicle is of prime importance in the calcification of cartilage. A group from the Hospital for Special Surgery, NYC is looking into the structure of bone mineral at the molecular level. A recent discovery was the importance of the lipid role in the calcium-phospholipid phosphate complexes associated with mineralization. Other grantees are exploring chemical and physical constituents of tendon with respect to mineralization.

A number of grantees are examining how the growth plate operates. One group is devising various techniques for separating zones of the growth plate to distinguish various biochemical changes. Grantees from the University of Pennsylvania are looking into cytoplasmic structures to understand functional differences in the plate. Another grantee at Montefiore Hospital in NYC is delving into the influence of proteoglycans on the biochemical structure and function of the growth plate.

There are several projects studying osteoporosis - a reduced bone mass disease of increasing incidence due to the extended life expectancy of our population. One grantee is undertaking a 20-year longitudinal study of calcium and bone metabolism in menopausal women. This study has shown that calcium absorption increases with increased calcium uptake, which then improves the calcium balance. Two forms of therapy are being evaluated in treating the disease - estrogen/androgen administration and calcium supplementation. A Texas Women's College grantee is conducting an X-ray study comparing bone quality changes 20 years after an initial X-ray. Another study is attempting to show that good health practices, proper nutrition and adequate exercise may prevent or delay osteoporosis. Still another is studying low bone mass in patients with diabetes mellitus. A grantee from the University of Southern California, in studying bone cells from patients with Paget's disease, has discovered unusual nuclear inclusions in osteoclasts. The inclusions resemble nucleocapsids of the paramyxovirus type which have been cultured from pagetic bone. Indeed the clinical course of Paget's disease is consistent with a slow virus disease.

A grantee at Johns Hopkins University, has obtained purified populations of normal mouse spleen cells which restore bone resorption when given to irradiated osteopetrotic mice. Utilizing the findings of these studies, a technique for marrow transplants for osteopetrotics is now being tested.

Other bone diseases of importance to this program are of genetic origin. We recently funded two new studies on diseases of this type. One is from an individual attempting to strengthen the fragile bones in Osteogenesis Imperfecta through electrical stimulation in a rat model. A second grantee is endeavoring to delineate the genetic heterogeneity which exists in Osteogenesis Imperfecta. In looking at genetic bone dysplasias, a Case Western Reserve grantee is trying to develop methods to culture human chondrocytes which maintains the phenotype *in vitro* and then examine proteoglycan structure and metabolism.

A group at the University of California, San Diego is looking at calcitonin and other calcitropic hormones to define their role in mineral

metabolism. Several new assays for studying diseased individuals have been devised. A member of the group has devised a marker for bone turnover utilizing vitamin K- dependent amino acid ∝ carboxyglutamic acid (Gla) levels in serum. Preliminary studies indicate that Gla levels are markedly elevated in patients with metabolic bone diseases characterized by increased bone turnover. This group also has been successful in developing in vitro models for studying hormone secretions which allow the simultaneous study of the secretion of parathyroid hormone and calcitonin. This unique assay model allows the study of complementary changes in hormone secretion during a variety of functional tests.

A group from Jewish Hospital, St. Louis, is looking into the metabolism of antirachitic steroids and applying this knowledge to human physiology and disease. Grantees from the University of California, Riverside were the first to detect by radioimmunoassay the presence in rachitic chick bone of a vitamin D - dependent calcium that tends to predominate in spongy bone and growth plate region. Another group at the University of Arizona is looking at vitamin D metabolites and their mode of action at the molecular level. This is accomplished by an assay for 1,25 dihydroxycalciferol and other vitamine D_3 analogs to look at target cells especially with regard to receptors and RNA synthesis. The findings of these groups may lead to better treatment for such diseases as renal osteodystrophy, hypoparathyroid disease, osteoporosis and vitamin D resistant rickets.

Cartilage. The metabolism of cartilage is being studied at various centers throughout the country. A group at Massachusetts General Hospital, Boston, looking at RNA, DNA, protein and glycoaminoglycans synthesis in osteoarthritic cartilage has found hyperactivity in the diseased cartilage. In addition, the group has discovered in articular cartilage a novel xylose acceptor which may be a secretory protein. Another individual in this group is delving into the effect of prostaglandins on articular cartilage. A grantee at Robert B. Brigham Hospital is examining diseased intervertebral discs for biochemical changes. Another grantee from Rush Presbyterian St. Lukes Hospital is studying the role of lysozyme which seems to be part of a proteoglycan aggregate in cartilage metabolism. The regulator of aggregates may be involved in proteoglycan breakdown in diseases such as osteoarthritis.

A grantee from Tufts University who last year found that vitamin C can modulate levels of phosphatase and sulfatases in articular cartilage has now shown that vitamin E can regulate sulfated proteoglycan metabolism and certain lysosomal enzyme activities in cartilage.

Joint Structure and Prosthetics. The Bone Diseases Program supports several projects related to the structure and function of normal and diseased joints. Several research efforts deal with general properties of joints; the largest concentration relates to hips and knees. The research approaches span a complete range from specialized techniques and transducers for experimental measurements to finite element theoretical modelling.

Internal joint pressure distribution may provide an understanding of normal and disease states as well as design information for replacement implant

devices. One NIAMDD grantee has developed a new ultra-thin transducer that is being utilized in dynamic measurements in cadaver and animal models. Another investigator at Massachusetts Institute of Technology is making similar measurements with an instrumented artificial femoral head and telemetry from the implant. The joint force distribution as related to associated cartilage thickness may partially explain the etiology of osteoarthritis. Reduced joint function as a result of degenerative disease is strongly influenced by the quality of joint lubrication. One investigator is analyzing the wear particles from synovial fluid specimens. This is useful for both the analysis of the progression of the disease and the selection of replacement materials. An NIAMDD grantee has shown specific proteoglycans to be responsible for the low friction in the synovial joints.

Joint mechanical property characterization has been advanced to the level of aiding practical orthopedic practice by investigators at UCLA and the Mayo Clinic. In one case a tapered, fitted leg device measures ligamentous stability in a quantitive way. The other research involves a complete multivariable analysis using gait, EMG, and other function tests to assess state of disease (as compared to normal subjects) with recommendations for treatment and type of implant replacement. Further extensions of joint testing are leading to mathematical models to simulate the normal joint, trauma to joints, and the various disease processes.

Several projects are supported on the development and testing of prosthetic joints. The developmental aspects include design, fabrication, and biomaterial components required for prosthetic devices. In vitro and in vivo evaluations are made to assess the device performance and subject adaptability.

In terms of biomaterial analysis, implant fixation receives the greatest funding and is likewise commonly identified as the biggest problem. Investigators at Clemson University are examining piezoelectric and electric stimulation methods for enhanced bone ingrowth. Another member of that research group is observing the influence of surgical procedures such as special cleaning and drying to improve the fixation with bone cement. Other investigators claim the free monomer residue from polymethylmethacrylate is the key to joint loosening.

In vivo testing is primarily applied to animals; however, some researchers are developing instrumented prosthetics for future implantations in humans. Biocompatibility and wear analysis are the primary aspect of these studies. One investigator has measured several mechanical properties of polyethylene socket material as related to grain size and oxide properties of the stainless steel ball. A uniform in vitro wear tester for simultaneous evaluation of several specimens has been developed at the University of California, Los Angeles.

Fractures and the Healing Process. There are many projects supported by the Bone Diseases Program related to the basic phenomena governing bone regrowth subsequent to fracture. Biochemical and biophysical studies are progressing on cellular and sub-cellular aspects of this problem. New bone labelling techniques are being developed. In more applied studies, an investigator at the University of California, San Diego has shown that more

flexible bone stabilizer plates reduce the later onset of osteopenia. A new healing detection transducer utilizes stress waves and the natural piezo-electric effect in bones to produce greater accuracy and less biohazard than possible with X-rays.

A substantial portion of the funding in the fracture area has been used to investigate improved healing using the recently developed methods of electric stimulation. Both direct current and magnetic field induction are utilized as stimulants. Some basic work has been started to observe cellular and extracellular effects from electric fields. One investigator is examining the induced change in cyclic AMP and its relation to collagen synthesis. Others are looking towards clinical application. Various animal experiments have characterized the required stimulation levels to produce improved healing and evaluate the nature of the new bone growth.

SKIN DISEASES RESEARCH AND TRAINING PROGRAMS

Two years ago the Senate Appropriations Committee recommended that the Institute initiate and support studies to determine the research needs and priorities in the field of dermatology. In response to this mandate the Institute awarded a contract to the University of Washington for such an analysis. The contract was awarded in April 1977 and will be completed in July 1979. The Coordinating Committee of this major effort has reached a stage where the major portion of the report concerning the prevalence and morbidity, the current status of dermatology research, and research needs in dermatology are in a form ready for final narrative editing and reproduction. In addition, the Committee is preparing summary statements including the appraisal of current manpower resources and needs for additional research personnel, and a general statement about the current level and needs for further development of technology. These major priorities of manpower and technology will be set forth in such a manner that priorities for the research effort can be developed from prevalence, morbidity, and cost of the important groups of skin diseases. The plan will be published in the Journal of Investigative Dermatology some time after the report has been received by the National Institute of Arthritis, Metabolism, and Digestive Diseases. The Institute expects to have a very useful document. The information developed indicates both the broad scope of research at present and important new areas of need.

Psoriasis. Psoriasis is a common disease, often disfiguring and disabling. In 1974 an effective treatment for psoriasis, orally administered 8-methoxypsoralen and long wave ultraviolet radiation (320 to 440 mm, UVA), known as PUVA, was reported. Although PUVA is an investigational therapy, an estimated 35,000 Americans received this treatment in 1978. Recognizing the need for a carefully controlled follow-up of PUVA-treated patients, the NIAMDD three years ago awarded a contract to the Massachusetts General Hospital, which arranged to subcontract to sixteen national university centers for collaboration in a five-year follow-up of PUVA-treated patients. In the Skin Diseases Program Area report for Fiscal Year 1978, it was reported that 1,200 patients studied had not demonstrated any gross abnormal pathological changes. In April 1979 the Massachusetts General Hospital, Harvard Medical School, and its 16 collaborators reported the risk of cutaneous carcinoma in patients treated with oral methoxsalen photochemotherapy for psoriasis (New England Journal of Medicine). They confirmed the beneficial effects of PUVA therapy in a large number of patients, and indicated that the therapy has the advantage of being effective, clean, and acceptable to patients, but that certain potential problems remain: the possible induction of cataracts; hematologic effects, such as abnormalities resulting from sister-chromatid exchanges; alterations of immune responses; aggravation or precipitation of specific diseases, such as lupus erythematosus and bullous pemphigoid; and chronic phototoxic effects on the skin and cutaneous carcinogenesis. The investigators reported that clinical problems related to the first three potential hazards have not yet developed. There have been occasional reports of bullous pemphigoid and lupus erythematosus but there is probably not a high risk of such complication for patients previously free of these conditions. In contrast, chronic phototoxic effects and cutaneous cancer formation may well represent an important

problem. In their evaluation of 1,373 patients they found 48 epidermal cancers in 30 patients. The incidence is 2.63 times that expected in a matched control population: patients previously subjected to ionizing radiation or who previously had skin cancers were at even higher risk; and in lighter-complexioned patients cancers developed much more readily than in those who tanned well. Of additional concern, the investigators observed a reversal of the ratio of squamous-cell carcinomas to basal-cell carcinomas, squamous-cell carcinomas becoming relatively more frequent and occurring on parts of the body not usually exposed to the sun. Since the sun is the primary stimulus for skin cancer in Caucasians, the reversal of the ratio and the alteration of anatomic distribution support the role of photochemotherapy in the causation of these cancers. The investigators conclude that psoralen photochemotherapy must still be considered experimental, but will probably find an important place as one of the treatments for severe psoriasis. They emphasize that the risk-benefit ratio must be further explored, the appropriate patient population must be identified, and methods of reducing the risk must be examined before the procedure can be considered for general use. The NIAMDD will continue to work closely with this most important national cooperative study.

Psoriasis/Dialysis. For the past several years there have been brief reports on the success of peritoneal dialysis on severe intractible patients with psoriasis who are resistant to all conventional and traditional forms of psoriasis therapy, including methotrexate and photochemotherapy. NIAMDD organized and sponsored a small Psoriasis/Dialysis Workshop in order to further focus on the potential significance of this new therapy. In April of 1979 a group of nephrologists and dermatologists met in Bethesda to consider the possibility that dialysis may affect the course of psoriasis. Despite a few outstanding successes the procedure must be carefully evaluated. There are few controlled studies. Investigators nevertheless expressed astonishment at the rapidity of recovery in some patients who had been so disabled by psoriasis that they had considered suicide. They had lesions over 75% of the body surface and often were unable to hold jobs. Of approximately 63 psoriasis patients reported at the meeting by investigators from around the world, 40 showed improvement following dialysis. Although no circulating factor has yet been identified, an investigator from Japan reported isolation of a protein with a molecular weight of 400 Daltons. Investigators in this country corroborated this finding, reporting molecules of the same weight bound to a larger protein and found in the blood only of psoriasis patients. Workshop participants expressed the opinion that improvement is not likely to be caused by placebo effect because patients who improved significantly had severe disease, unresponsive to every existing therapy. On the other hand, the only double-blind, controlled study reported at the meeting explored the effectiveness of hemodialysis in psoriasis patients and had negative results. Participants resolved to have another meeting concerning psoriasis and dialysis which will include other interested investigators. Any major national effort will require more convincing pilot studies. A nation-wide computer hook-up will be established in order to store data on psoriasis-dialysis therapy, and reporting forms were provided to conference participants.

Severe Acne. In the February 15, 1979 issue of the New England Journal of Medicine a major breakthrough in the treatment of the most severe forms of acne was announced by the National Cancer Institute in cooperation with the NIAMDD. The new treatment produced complete clearing in 13 out of the 14 patients studied. For most individuals acne is a common benign inflammatory skin disorder. However, it is distressing for those who are afflicted more severely because of its usual location on the face. For most individuals the disease usually disappears after months or years and therapy, although not curative, can achieve satisfactory suppression of activity in the majority of patients. A small number of acne patients with severe and extensive disease show little or no response to intensive therapeutic efforts, including the use of high doses of oral tetracycline, dapsone, prednisone, and, in women, estrogen. Those patients with large inflammatory suppurative nodules involving not only the face but also the back and chest suffer more than social embarrassment. The lesions are tender and often purulently exudative and hemorrhagic. Scars are often disfiguring. The report by NCI Intramural investigators and NIAMDD grantees in the issue of the Journal that treatment of severe and treatment-resistent cases of cystic and conglobate acne with oral 13-cis-retinoic acid (a synthetic isomer of the naturally occurring all-trans-retinoic acid) produced dramatic clearing of the disease in 13 out of 14 patients treated is a potentially important advance in the management of such patients. The mechanism proposed for the action of this drug is its marked inhibition of the synthesis of sebum, the secretory product of the sebaceous gland, which according to NIAMDD grantees is an indispensable link in the pathogenetic sequence leading to inflammation in acne. The New England Journal article cautioned readers that long-term trials are needed to learn whether this treatment has long-lasting benefit or harmful effects. The complete resolution of severe acne and the apparent persistence of this markedly beneficial effect 12 to 20 months after discontinuance of the drug are significant events. The NIAMDD will continue to work closely with investigators in the extension of these preliminary observations to larger-scale studies.

Vitiligo. In July 1977 Congress recommended that the NIAMDD substantially expand its research on vitiligo. Vitiligo is a common skin disorder characterized by the destruction of pigment cells. The disease affects approximately two million Americans and produces severe cosmetic disfigurement. Although vitiligo patients with light skin are often upset by this condition, patients with dark skin may suffer serious psychological and social handicaps. There are numerous medical problems that can be associated with vitiligo. For example, in a high percentage of vitiligo patients there are abnormalities in the pigment cells of the eye. It is possible that some patients with loss of vision due to inflammatory processes, such as uveitis or iritis, may have vitiligo in the pigment layers of their eyes. In April 1979 the NIAMDD announced the award of a four-year grant for the purpose of expanded vitiligo research. The grant, totaling approximately two million dollars, was awarded to a consortium comprised of Yale University, Howard University, Bryn Mawr College, the University of Pennsylvania, the University of Massachusetts, and the Massachusetts Eye and Ear Informary. Because of the many effects of vitiligo and its association with other diseases, the NIAMDD consortium grant will cover several areas of study. A clinical center for vitiligo

patients will be established and the relationship of vitiligo to other diseases such as the auto-immune disorders and melanomas, and the treatment of such will also be undertaken. Howard University Hospital has established a clinical center for the diagnosis and treatment of vitiligo patients. Since the First International Workshop on Vitiligo that was held at the NIH in March of 1977, there has been a steadily increasing number of applications for research grants into this disease. The NIAMDD will closely monitor the activities of this major research initiative and cooperate in planning for future efforts in vitiligo research.

Research and Training Support. The Skin Diseases Program is responsible for approximately two-thirds of the total NIH support for basic and clinical studies of the cutaneous system in normal and disease states. The total research budget for FY 79 was $5.83 million, and the training budget was $735,000. Currently there are eight recipients of Research Career Development Awards, and six individuals being supported by Clinical Investigator Awards. Three individual fellowships and thirteen institutional training grants were supported in FY 79.

MULTIPURPOSE ARTHRITIS CENTERS PROGRAM

Overview

The Multipurpose Arthritis Centers Program has grown to a current level of twenty-four Centers in eighteen states from New Hampshire to Hawaii. Fifteen Centers were first funded in FY 1977, seven more in FY 1978, and two in FY 1979. Three additional applications were received for the February 1, 1979 deadline and are now in review.

During FY 1978, twelve Centers submitted supplemental applications requesting funds for "developmental and feasibility studies." These studies were designed to support exploratory research, including pilot studies and research by young investigators. Of the fifty-four individual projects reviewed by an Institute Special Review Committee, twenty were awarded. Fifteen of these projects dealt with biomedical research, and the remainder were in the areas of research in education and community demonstrations.

In FY 1977, $2.5 million was available for the Arthritis Centers Program. The fifteen funded Centers received an average of $167,000 (total costs) ranging from $61,000 to $200,000 for the initial year. For FY 1978, $5.2 million was available and the average award to a Center was $234,063. During FY 1979, approximately $7.3 million is available. These funds are committed to funding both the existing 24 Centers and a Congressionally-mandated one-time award to the Center at the University of California in San Francisco, in honor of the late actress Rosalind Russell.

Activities in the Centers

These encompass a wide range of research disciplines and interests from biomedical to social science. Presented here is a selected sample of such activities.

The Arthritis Center grant at Washington University is supporting an investigation of the effect of a biologically active vitamin D metabolite in reducing the bone mass loss associated with the steroid therapy frequently used in treating arthritis. In a small series of patients, the vitamin D metabolite was shown to be beneficial. These studies are continuing in concert with an investigation on the effect of steroids on vitamin D metabolism in humans.

In the Center at Indiana University, investigators are studying proteoglycans in articular cartilage. They have already shown that disuse of a joint rapidly inhibits proteoglycan aggregation in human articular cartilage. These observations have been confirmed in dogs in which a knee was immobilized by casting. Although there was a rapid onset of defective matrix organization, the phenomenon was completely reversible after removal of the cast. Future studies will attempt to differentiate between the effects of immobility versus non-weight bearing on the organization of proteoglycans in articular cartilage matrix. Other studies at this Center are focussing on Amyloid Serum A (ASA): a substance found to be markedly elevated in the serum of many patients with rheumatoid arthritis or infections as well as cancer. Initial findings indicate a strong similarity between ASA isolated from both humans and a

mouse model. Future work will be concerned with determining the effect that this material has upon the immune system.

The Center at the Medical University of South Carolina is attempting to find a new method for the early diagnosis of Raynaud's phenomenon and scleroderma. It was noted that serum from patients with scleroderma possess a factor which breaks chromosomes. Investigators at this Center are developing a sensitive procedure, sister chromatid exchange, to detect such chromosome damage. Sister chromatid exchange will be examined to determine their prognostic usefulness in identifying persons at risk for developing scleroderma and/or predicting the severity of the disease.

Clinical trials are also a feature of Arthritis Center research activity. The Stanford University Center has assembled a group of rheumatologists to perform prospective clinical trials to evaluate treatments for arthritis. The first study is testing the comparative effectiveness of several non-steroidal anti-inflammatory agents. In double-blind studies, patients with rheumatoid arthritis or ankylosing spondylitis (a chronic progressive disease of the spine resulting in immobility) receiving six drugs in random sequence. Each treatment is followed by a functional evaluation. Preliminary data indicate that the non-steroidal anti-inflammatory drugs are not, in fact, interchangable. Some are more effective in the treatment of rheumatoid arthritis, while others are more beneficial for ankylosing spondylitis.

In the Centers at Stanford University and the University of Arizona, cost-of-illness studies are underway. Medical costs on a fee-for-service private physician practice are being compared to those in a health maintenance organization, and to those in a university clinic. These studies also include an assessment of health outcomes in patients with arthritis. Similarly, the University of Alabama is determining the social and economic impact of rheumatoid arthritis on its patients in order to provide a baseline for evaluating the impact of medical and other interventions in that disease.

A number of Centers are testing the value of the team approach to arthritis care. The team is composed of a physician, a nurse, a physical therapist and/or an occupational therapist, and a social worker. The team care concept of treatment is being compared to the more usual model of medical care in which physicians evaluate and manage the patient and then prescribe for services to be provided by allied health professionals.

Arthritis Centers are also active in the production of audiovisual aids for use as educational tools. Three films have been completed by the Center at Washington University in St. Louis. These films feature a presentation by a professor, acknowledged to be an effective educator, who traditionally presents the introductory lecture in rheumatology to medical students. These films are being presented to a portion of the medical class at four medical schools with Arthritis Centers: Dartmouth, the University of Arizona, the University of Michigan and Washington University. The same professor will also lecture to the remaining members of the same classes with the same instructional content as was present in the films. The students will then be tested both for long-term and short-term recall. This study will thus provide a basis for deciding the value of filmed lectures in the education of students in various health professions.

Many of the Centers are evaluating continuing medical education programs for practicing physicians. An innovative approach to continuing education is being explored by the Arthritis Center at the University of Michigan. A member of that Center is identifying persons who are though to be "educational influentials" in community hospitals. These persons are physicians who are generally approached by other colleagues when they want consultation or advice about difficult cases. These "educational influentials" will be instructed in the latest rheumatological practice by members of the Center and thus will act as resource physicians in this specialty for other physicians in their local hospital. At the same time, physicians in other community hospitals will receive lectures and rounds from Center staff in the traditional manner. Physicians in all of these test communities have been recruited to maintain office logs to record each office visit and note the diagnosis and prescription. The Center will then analyze these logs to determine if and how the practice of medicine has changed in any of these communities.

Another new program in continuing medical education, being developed at the University of Alabama Center in Birmingham, is a system which provides 24-hour-a-day medical instruction by tape recordings accessible through long-distance toll-free telephone lines. Diagnostic and therapeutic information is available on specific programs in the rheumatic diseases. This system has already been approved for Category V Continuing Medical Education credit. Each year, callers will receive a certificate giving the total number of credit hours they have acquired.

More than half of the Centers are also involved in the education of Allied Health Professionals, since most of these individuals rarely receive training which has prepared them to deal effectively with arthritis patients. To counteract this deficit, curricula and audiovisual materials are being developed for inclusion into undergraduate training programs. For those Allied Health Professionals already graduated, there have been seminars, lectures, and workshops. In some cases, Allied Health Professionals have received practical instruction from assignment at the Center itself.

Center Coordination

In March 1979, the Second Annual Center Directors' Meeting was held on the NIH campus. During that meeting, representatives from the Centers presented summaries of their ongoing activities and discussed plans for cooperative experiments and activities between and among Centers.

SPECIAL PROGRAMS IN ARTHRITIS AND RELATED DISEASES

The following important activities are being developed to address certain specific features of the National Arthritis Act of 1974, as amended in 1976, and several recommendations of the National Commission on Arthritis and Related Musculoskeletal Diseases as contained in its Arthritis Plan submitted to the Congress in April 1976.

Arthritis Epidemiology Program

The objective of the newly organized Arthritis Epidemiology Program is to encourage and support research concerning the incidence and prevalence of arthritis and related diseases, what causes them and who gets them. The need for these studies is viewed as particularly compelling now because "the results of biochemical research suggest that susceptibility to certain of these diseases may be genetically linked to other measurable genetic characteristics," according to the 1978 Annual Report of the National Arthritis Advisory Board. Meeting this need has been hampered by a shortage of trained arthritis epidemiologists and a lack of funding opportunities.

The Arthritis Epidemiology Program Office is working on the following activities to foster arthritis epidemiological efforts. A literature review of arthritis epidemiology is in preparation. A report on epidemiological activities in the Multipurpose Arthritis Centers is in progress. In addition, a workshop on arthritis epidemiology is being planned. The thrust of these efforts is to generate research interest and proposals which utilize the scientific discipline of epidemiology to study the distribution, natural history, and determinants (risk factors) in those rheumatic diseases that are likely to yield important information as to etiology by this approach. Ankylosing spondylitis, Reiter's syndrome, systemic lupus erythematosus, rheumatoid arthritis and osteoarthritis are among those diseases with a likely potential for yielding such new knowledge.

Arthritis Data Systems Program

The recently organized Data Systems Program Office has been created to foster the systematic acquisition and storage of information about arthritis and related musculoskeletal diseases patients; and to encourage efforts to relate the characteristics of the patient, the disease, and medical intervention to outcome. The overall purpose is to enhance understanding of arthritis and improve the clinical care of affected patients.

To aid NIAMDD in the implementation of the above objectives, a multidisciplinary Data Systems Subcommittee of the Arthritis Interagency Coordinating Committee has been formed. This advisory group will:

a. review ongoing programs and propose additional activities,

b. recommend priorities and standards for evaluation, and

c. seek to ensure that adequate guarantees of privacy, usefulness and accuracy are built into Arthritis Data Bases.

Presently there is a prototype for an arthritis data system, the American Rheumatism Association Medical Information System (ARAMIS), which is in its second year of funding by NIAMDD. ARAMIS is a multi-institutional collaborative project for the development of an information system and database addressed to the care of patients with rheumatic diseases. Several centers, including some of the Multipurpose Arthritis Centers, now contribute, or are planning to contribute, detailed, uniform clinical data to a central computing facility in Stanford University where the data are maintained in parallel data banks. The data may be accessed through a national communication network from any location. ARAMIS utilizes a data base system (TOD) which provides for the entry, storage, retrieval and analysis of time-oriented clinical information in an interactive mode.

Arthritis Community Programs

Through effective coordination between appropriate federal agencies, the Community Programs Office has been developing several programs leading to model community demonstrations and the mobilization of services for patients with arthritis.

A Community Programs Subcommittee of the Arthritis Interagency Coordinating Committee has been formed with broad representation and under the Chairmanship of the Commissioner of the Rehabilitation Services Administration. This group or special subgroups have met four times this year to date.

Three separate initiatives have been undertaken.

1. Child Health Initiative. Expert pediatric rheumatologists have emphasized that holistic services are needed for the child with arthritis. Participating agencies in planning will include crippled children's program, education agencies, rehabilitation services, pediatric rheumatology centers and others. New knowledge will be generated on program strategies, and on the effects such program collaboration can have on prevention of physical and psychosocial handicaps with potential for personal and economic dependence.

A discussion paper was prepared. An ad hoc Advisory Committee was organized to discuss the specific needs for such an initiative. The group expressed their accord with the proposed effort and provided the program with advice and suggestions. The Community Programs Subcommittee discussed these plans and provided additional advice. In addition, the Community Program Officer has met with program officers throughout agencies of DHEW whose program may have mutual interest in activities covered in this initiative.

A Guidelines Discussion Paper was prepared for consideration by Bureau of Education for Handicapped as possible lead agency for demonstration grants and by the support agencies. Various programs within the Bureau are being contacted. Funding potentials are now being explored.

2. Working Adult Initiative. The objective of these demonstrations will be to develop strategies for programs which can help prevent absenteeism and the need for early retirement because of disability due to arthritis or similar crippling conditions.

3. Aging. This initiative will explore with appropriate federal agencies the possible development and delivery of special services needed by the elderly with arthritis, as part of the senior centers program or other exploratory demonstrations. A discussion paper regarding ambulatory health care services for arthritis patients, as part of senior center programs, is now being analyzed by Administration on Aging.

Each of the above initiatives will encompass a variety of individual interagency activities, i.e., screening; treatment; evaluation/research; public, patient, family education; counseling; public education; vocational assessment and training; materials development; health systems delivery evaluation; and others.

Arthritis Education Programs

The National Arthritis Act and the Arthritis Plan directed the establishment of an information service to serve as a repository or broker for the nationwide flow of arthritis information designed to help health professionals and educators in their interaction with patients, their families and the public. The Arthritis Information Clearinghouse was established in the Education Programs Office to carry out many of these activities.

The NIAMDD has appointed an Advisory Group comprised of rheumatologists, educators, library scientists and other health professionals to help the Clearinghouse in the effective accomplishment of its goals. The Group has met twice within this reporting period, and provided recommendations.

The Arthritis Information Clearinghouse provides the following products and services: Arthritis Clearinghouse Bulletin to be published periodically to alert users as to new developments; Clearinghouse Thesaurus; Catalogue of Accessions; bibliographies ranging from the psychological factors in arthritis to pain management; Guide to Audiovisual Materials; Fact Sheets; and Directories such as on Organization Resources.

Associate Director's Report for the Annual Report
of the Digestive Diseases and Nutrition Cluster

The Report of the National Commission on Digestive Diseases

The National Commission on Digestive Diseases completed its report and presented it to Congress on January 31, 1979. The long-range plan contained in the report had 23 recommendations. The cornerstone of this long-range plan was increased acquisition of new knowledge through research. This was proposed as the most cost effective means of improving treatment of digestive diseases and ultimately developing means of preventing these diseases.

The Commission recommended that increased funds be provided to support basic and clinical research. It also recommended increased funds to support research into the best techniques for rendering high quality, cost effective health care taking advantage of the latest research findings. The Commission recommended the individual research grant as the most effective means of supporting research, but recognized the value of consortia of scientists and of research centers when a special research objective required the expertise and infra structure not available to a small research team. The Commission identified specific areas that appeared to be promising and important areas for research, and it identified subjects that might be the bases of workshops to stimulate research in specific areas. It also recommended increased funding to train investigators for the difficult and complex problems of research in digestive disease.

The Commission recommended the establishment of a national digestive disease education and information clearinghouse to gather existing material on digestive diseases, prepare needed material, evaluate all material, and arrange for distribution to the public, to patients, to physicians, and to other health professionals. This clearinghouse was to be established through NIAMDD. The Commission also recommended the strengthening of professional education in digestive diseases in medical schools, through teaching awards, and the provision of faculty from other institutions to supplement teaching in institutions that lack adequate staff. It also called attention to the need for research and training in the psychosomatic aspects of digestive diseases.

The Commission called for a program to standardize terminology and diagnostic criteria in digestive diseases. It emphasized the need for more accurate and comprehensive data on the epidemiological patterns of digestive diseases and recommended funds to obtain such data, and to train epidemiologists in digestive diseases.

It recommended that a national digestive diseases advisory board be set up to review, advise on, and facilitate coordination of Federal activities in digestive diseases.

The Digestive Diseases Coordinating Committee

The Digestive Diseases Coordinating Committee worked closely with the Digestive Diseases Commission supplying it with the information it requested. Individual institutes and agencies identified for it ways in which they might improve the performance of their missions relative to digestive diseases. After the completion of the Commission report, the Coordinating Committee examined the final recommendations, and has begun implementation of them. A Subcommittee is already planning for the implementation of the recommendation concerning standardization of the nomenclature and diagnostic criteria for digestive diseases.

In a series of meetings, the institutes and agencies that are members of the Coordinating Committee have been describing their missions, organization, and current activities. A report on the activity of each institute and agency has been prepared. As result of the interaction in the Coordinating Committee, cooperative activities between institutes and agencies have been initiated.

DIGESTIVE DISEASES PROGRAM AREA
(1978-1979)

The Digestive Diseases Program Area is responsible for the extramural support of grant awards and contracts pertaining to certain organ systems of the gastrointestinal tract. Digestive Diseases include disorders of the esophagus, stomach, and small and large intestines. Major digestive diseases include esophagitis, peptic ulcer, diverticulitis, infectious diarrhea, ileitis, ulcerative colitis, and malabsorption. The known causes include infections, metabolic disorders, genetic defects, neuromuscular disorders, toxins, and reactions to the life stress.

Digestive diseases constitute a health problem of great magnitude. Using several natural indices it is found that one out of every six illnesses in our population arises primarily from diseases of the digestive tract. The National Center for Health Statistics has found that nearly 13 million Americans have chronic digestive diseases; such conditions together constitute the number one reason for hospitalization in this country. Digestive diseases are found in all age groups, are often chronic, and may lead to many social and emotional problems.

During the period of October 1, 1978 through September 30, 1979 approximately 175 research grants, one specialized center of research, 19 training programs, 27 career awards, and 12 individual fellowships have been supported in the Digestive Diseases Program Area. In addition, several contractor efforts were also active which included a clinical trial and applied research and development projects.

A. Research Activity Supported By Grants

During the FY 1979 much of the research activity supported by grants was concerned with the basic science aspects of digestive diseases. A greater part of the total research, however, was disease oriented, the results of which were relevant to the elucidation and understanding of the diseases process. Of the considerable amount of data and information reported by the grantees during the past year, only a few examples will be cited as representative of the progress that has been made in the Digestive Diseases Program Area.

1. Peptic Ulcer Disease

a. Peptic ulcer disease is still considered one of the major and more prevalent of the many digestive diseases. Fortunately, however, it is highy amenable to treatment, whether by the use of antacids or the new drug cimetidine. Treatment of duodenal ulcer with an H_2 receptor antagonist, such as cimetidine results in healing of duodenal ulcer in over 80% of patients within 6 weeks. However, cessation of treatment after 6 weeks is associated with an appreciable relapse rate, going from 40% within 3 months to over 80% within 12 months. Of course, patients remain asymptomatic, if cimetidine therapy is not discontinued after 6 weeks. Currently, however,

prolonged use of cimetidine is not recommended. Relapse occurred sooner and more frequently in these patients with the longer history of ulcer disease.

These reports have aroused considerable concern and interest in the technical area of cytoprotection or tissue resistance to those many factors that have been shown to produce tissue destruction. Of course of equal importance are those factors involved in tissue regeneration. Theoretically, an ulcer can develop if either regeneration decreases or destruction increases sufficiently to result in an imbalance. The destructive factors that are being studied include; acid, pepsin, aspirin, ethanol, bile, and "stress". Less is known about alterations in the regenerative forces that could lead to ulcer formation.

Recognition of the complexity of the problem of gastric and duodenal ulcer formation and prevention, together with the ubiquity of this disease process has resulted in a significant degree of research activity supported by the Digestive Diseases program of NIAMDD. This may also be a reflection of the technology that is available to investigators. Nevertheless, for the current reporting period, there are 47 individual grants supported for the study of ulcerogenesis. Of added significance are the equally large number of research projects that are under the aegis of the Peptic Ulcer Center also supported by NIAMDD. Considerable progress has been made towards elucidating the mechanisms and/or factors that contribute to the formation and prevention of ulcer erosions. Rather than a summary of the progress of these many projects, or even a selected few, since many are of equal importance, a representative list of the research grants should suffice to convey the scope and breadth of currently active research.

Damage and Repair of the Gastric Mucosal Barrier
Principal Investigator (P.I.) - Davenport, H.
Mechanisms of H+ and CL - Transport by Gastric Mucosa
P.I. - Forte, J.

Pepsinogen Polymorphism in Man
P.I. Samloff, M.

Neurogenic Control of Gastroduodenal Function
P.I. - Brooks, F.

G.I. Mucosal Barrier in Health and Disease

P.I. - Silen, W.

Gastric Mucosal Integrity: Intracellular Hypoxia
P.I. - Bowen, J.

Cyclic Nucleotides in Gastric Acid Secretion
P.I. - Ekblad, E.

Reflux of Bile Acid As A Factor in Gastric Disease
P.I. - Ritchie, W.

Biochemistry of Lipids In Normal and Ulcerated Stomachs
P.I. - Horowitz, M.

Psychobiologic Mechanisms of Stress Ulcer in the Rat
P.I. - Ackerman, S.

G.I. Hormones and Other Factors on Growth of G.I. Mucosa

P.I. - Johnson L.

Gastric Zymogens, Proteases and Inhibitors

P.I. - Glick. D.

2. Diarrheal Diseases

A very significant contribution to the study of diarrheal diseases has been the conceptualization and experimental verification of a basic model for secretion. This model has emerged from many man-years of intensive research by several Institute grantees. Although worked out to explain intestinal and colonic secretion, this model may have even wider applicability because emerging data suggests that a similar process may be operative for the stomach, tracheal mucosa, cornea, salivary glands and pancreatic exocrine cells when any of these tissues are stimulated by their appropriate secretagogues.

The basic features of this model for electrolyte secretion, developed by experiments in the rabbit colon, are as follows: The essential step takes place at the serosal membrane of the colon cell where the sodium concentration in the extracellular fluid is much higher than inside the colonic cell, allowing sodium to move into the cell over a "downhill" gradient This process carries the chloride ion into the cell from a low extracellular concentration into a high cellular concentration ("uphill" gradient). The gradient for Na^+ across this membrane is maintained by an active process, referred to as the Na^+ pump that keeps pumping Na^+ from the cell back into the extracellular fluid to be recycled. Meanwhile, back in the colon cell the Cl concentration becomes very high and can therefore move "downhill" into the gut lumen (across the mucosal barrier) by diffusion. Various secretagogues, which increase intracellular cyclic AMP, which in turn results in an increase in intracellular calcium activity, appear to stimulate this chloride exit. This results in an increase in the Na^+ and water moving into the lumen or net secretion. Calcium may stimulate secretion by the simple process of increasing the permeability of mucosal membrane, thereby allowing an increased diffusion of the chloride into the lumen.

This very basic work, dealing with physical-chemical principles, molecular properties of membranes, etc. has major clinical relevance to diarrheal disease states such as cholera, in which secretion is abnormally increased. Several years ago, other Institute grantees showed that D,L-propranolol a β- blocking agent used primarily in cardiac disease, could inhibit secretion caused by bile acids in the rabbit colon. The mechanism for this action seemed to be through the inhibition of adenylase cyclase, a key enzyme in cAMP formation. Ileal secretion, however, was not inhibited.

Just this year, using another experimental model (rat ileum and colon), other grantees have shown that D,L-propranolol can also inhibit ileal as well as colonic secretion caused by cholera enterotoxin. Both of these studies tie in very well with the mode for secretion postulated above; i.e. increased cAMP increasing secretion via its effect on permeability of the mucosal membrane. They also have important clinical implications in the treatment of patients with diarrhea, which can now be explored.

3. Inflammatory Bowel Disease

a. Studies have revealed the presence of small RNA viruses, cytomegalovtus, and cell wall defective mycoplasma in ulcerative colitis (UC) tissue. Other small RNA viruses, M. kansasii, and cell wall defective pseudomonas - like bacteria have been associated with Crohn's disease (CD). However, the significance of infectious agents in these diseases remains unclear. A prospective study was undertaken to determine if cultivated cytopathic agents were similar, were viral or nonspecific macromolecular substances, were disease specific, and could be transferred to rabbits by intavenous route. Surgical specimens from UC patients, and CD patients, colon carcinoma patients and patients with other gastrointestinal diseases were appropriately prepared and processed for the various procedures that were employeed such as inoculations and immunizations. To date results that have been reported show that cytopathic agents were isolated from each of 28 CD patients, 24 of 25 UC patients, 8 of 9 colon carcinoma patients, and from none of the 24 patients with other gastrointestinal diseases. Guinea pig antibody against the CD isolate of one patient in California inhibited the growth of a CD isolate obtained from a patient in Vermont. Human CD antibody inhibited the growth of the California isolates but not of the Vermont isolates. Serial passage of the agents was successful for more than 15 passages. Cytopathic effect was inhibited by specific antibody, Intravenously inoculated rabbits did not develop CD or UC. The investigators conclude from these results that 1) isolated viruses may not be identical,2) the cytopathic agents are replicating agents and are not macromolecular substances since they are inhibited by specific antibody and can be serially passaged. 3) Cytopathic agents have been found in CD, UC, colon carcinoma and not in other conditions, 4) rabbits inoculated by the intravenous route have not developed CD or UC.

b. Pseudomonas - like cell wall - deficient (CWD) bacterial variants have been isolated from Crohn's disease (CD), but not ulcerative (UC) tissues. A NIH supported grantee studied sera from patients with CD or UC and control subjects for the presence of antibody against the following organisms: 2 reverted CWD strains from patients with Crohn's disease and known strains of pseudomonas, E. coli and bacteroides. Presence of antibody in sera was studied by indirect immunofluroescence. Results showed that 16 sera from 22 CD patients (72%) had antibodies for one or both revertant CWD strains. In comparison, 5 sera from 23 UC patients (22%) and 2 sera from 10 control subjects (20%) showed similar results, only in patients with CD was there positive correlation between the clinical activity of disease and the degree of the response to the revertant CWD strains. Significant responses occurred in 20-30% of UC and

CD patients and control subjects against known strains of pseudomonas and E coli. All sera showed intense responses to bacteroides. These results show that 20% of the normal subjects and UC patients have been exposed to CWD like organisms, whereas in Crohn's disease, the frequency is significantly greater. Whether this relationship, however, is primary or secondary remains to be determined.

B. Research Activities Supported by Contract

1. National Cooperative Crohn's Disease
(Phase III)
Contract No. N01 - AM - 2-2210
University of Colorado, Denver, Colorado
Total Duration - 2 years Total Costs - $563,000

The Crohn's Disease Study is now in the terminal stage. All patients accepted into the study have completed a course of therapy that was selected as prescribed for a randomized clinical trial. The data is now being analyzed and subsequently, appropriate reports will be written to reflect the results of the study. This trial has three primary objectives, as follows: 1) compare the efficacy of prednisone alone versus the combination of prednisone and sulfasalazine in the treatment of acute symptomatic Crohn's disease; 2) to determine the "prednisone sparing" effect of sulfasalazine with those patients who have responded successfully to therapy in accordance with (1): 3) if sulfasalazine fails to demonstrate a "prednisone sparing" effect, to determine whether it will sustain a prednisone induced remission.

This study has been conducted under the direction of Drs. John Singleton and Fred Kern, Jr. of the University of Colorado.

2. Research and Development Studies in Gastrointesinal Endoscopy

Contract N01-AM-5-2211
Title: Endoscopic Control of Continued Massive Gastric and Duodenal Bleeding in a General Hospital Population

Institution: University of Washington, Seattle, Washington

Duration: Four years

Contract Amount: $872,066

This contract study is being supported to study the control of gastrointestinal bleeding by endoscopic systems involving laser coagulation, electrocoagulation, suture clips, and tissue glues. To date, significant progress has been made. Biocompatible tissue glues and suture clips have failed to stop severe bleeding in the ulcer model that was developed during the early phases of the contract. Only one modality has been found to be promising for the safe and effective control of massive bleeding. This is the argon laser used in conjunction with pressurized carbon dioxide. Currently, a pilot controlled clinical trial for approximately 100 patients

at the University of Washington is being conducted. The data obtained from this study will serve as the basis for a larger multicenter controlled trial.

The studies at the University of Washington are under the direction of Drs. Fred Silverstein and Cyrus Rubin.

LIVER DISEASE PROGRAM AREA

In the United States, liver disease is a major cause of death and accounts for enormous human misery. Many lay persons immediately think only of viral hepatitis or of alcoholism and cirrhosis when "liver disease" is mentioned. Although it is true that viral hepatitis and alcoholic cirrhosis affect thousands of individuals and are major health hazards, there are many other forms of liver disease which kill patients in all age groups. These include hepatitis due to drugs, chemicals, and environmental pollutants; biliary atresia and other serious liver diseases of newborns and children, and liver cell cancer and cirrhosis due to other causes. Throughout the world, the major liver disease, Schistosomiasis, is due to a parasite which accounts for millions of patients in Brazil, Puerto Rico, Egypt, and the Orient. Although this Institute does not support all of the liver-related research activities of the NIH, it does support the major share. Therefore, this report touches on only selected examples of important new findings.

Highlights of the research activities of grant-supported investigators will be found in the first section. Contract supported activities are reported on in the second section and a summary of an evaluation of the career program is found in the last section.

I. Research and Center Grant Activity

A. Protein Metabolism

A number of grantees are looking at protein metabolism in the liver and attempting to delineate the molecular mechanisms involved. For example, Dr. Leonard S. Jefferson at Pennsylvania State University has shown that the messenger RNA coding for albumin is decreased in concentration in the diabetic animal. Its level can be restored to normal following insulin administration. Growth hormone also has a similar effect on the specific mRNA for albumin, whereas the decrease in albumin synthesis in thyroidectomized animals is by a different mechanism. Dr. David Shafritz at Albert Einstein School of Medicine, a member of the Liver Center there, is also looking at how various altered physiologic and disease processes alter protein synthesis by the liver. By the use of specific probes for albumin mRNA (use of labeled cDNA prepared by cloning bacterial systems), he has shown that fasting causes a suppression of albumin synthesis by the release of a large proportion of albumin mRNA from polyribosomes into the cytoplasm. Refeeding amino acids restores the mRNA to the membranebound polysomes. This same approach (the use of specific and sensitive molecular probes) is now being extended and utilized to study the basic mechanisms of how other proteins synthesized by the liver, such as collagen, are affected by different agents causing liver injury. Thus, three animal models will be utilized in exploring how chemicals (carbon tetrachloride), liver parasites (schistosomiasis), and viruses (Hepatitis B virus) affect the protein machinery of the liver at the molecular level. Dr. Colvin

Redman, at the New York Blood Center, is looking at albumin secretion in rat liver hepatomas which appear to be unable to secrete albumin into the bloodstream in vivo. However, in vitro, no defect is apparent and the reason is being further explored.

B. Hepatic Encephalopathy

Severe liver disease is almost always associated with brain dysfunction. Chronic cirrhosis can cause a progressive blunting of intelligence progressing to stupor, movement disorders and dementia. The causes of the neurological deterioration are unknown, but their identification and effective management would substantially reduce the disability of patients with liver disease. Many toxic agents have been implicated in hepatic coma, the most common being ammonia, and "false neurotransmitters" such as octopamine. Dr. Thomas Duffy, Cornell, believes that ammonia is the leading candidate for this dubious honor because of some current studies. Through the use of ^{13}N-ammonia, he showed the highest rate of brain uptake of ammonia in patients with the highest plasma ammonia concentrations; i.e. those with liver disease. With three-dimensional imaging, his group is now trying to determine if the areas of the brain showing the greatest uptake correlate with areas exhibiting damage. Using an animal model, he has shown a great sensitivity to very small doses of ammonium salts and has shown that ammonia interferes with important processes in nerve cells including chemical communication, energy production, and possibly intracellular hydrogen transport. Offering evidence favoring the opposite view, Dr. Bahjat Faraj at Emory is showing an accumulation of tyrosine and tyramine in the brain of a dog model of the disease and this is accompanied by a severe depletion of brain catecholamines. Dr. Josef Fischer at Massachusetts General Hospital and now at the University of Cincinnati introduced the working hypothesis that the blood-brain barrier is responsive to the plasma concentration of the branched chain amino acids in an attempt to maintain a constant concentration of these amino acids within the central nervous system. In preliminary unpublished reports, this investigator has been able to produce a coma-like state in dogs by injecting aromatic amino acids (phenylalanine and tryptophan) into the carotid artery of the dog which produced an increase in their levels in the cerebrospinal fluid. The coma is prevented only when the branched-chain amino acids are added to the injected solution.

C. Heme Metabolism

Newborn infants often have significant elevations of serum bilirubin (known as neonatal jaundice) shortly after birth. This breakdown product of heme, which is the prosthetic group of hemoglobin, is normally conjugated by the liver into mono and diglucuronides which are execreted into the bile. Since this conjugation mechanism may develop more slowly in some infants, hyperbilirubinemia and even jaundice is commonly seen. Since its introduction about 10 years ago, phototherapy with broad spectrum fluorescent lights is being used to treat the hyperbilirubinemia seen in about 15% of the newborn population. This broad spectrum light exposes the infants to some

ultra-violet and infra-red radiation, which is potentially harmful. Therefore, an Institute grantee is trying to determine which wavelengths are actually responsible for the degradation of the bilirubin so that exposure could be confined to these wavelengths. Dr. Gerard Odell, University of Wisconsin, is exposing bilirubin solutions in-vitro to monochromatic light from an argon laser at selected wavelengths and studying the decay rate of bilirubin. He is also isolating induced photoproducts which can be studied for potential toxicity. In vivo testing of these wavelengths is being performed on a genetically jaundiced Gunn rat in which the bile is collected and analyzed. Such studies may permit the eventual design of a phototherapy unit for clinical use that would minimize or eliminate the potentially harmful effects of broad spectrum fluorescent lights.

At the Liver Center, University of California at San Francisco, Dr. Maria Correia and her colleagues are studying the "free" heme pool in the liver cytoplasm. The availability of utilizable heme in the liver is finely tuned to its synthesis and degradation. Heme itself plays the key regulatory role in both its synthesis by repressing the key rate-limiting enzyme (8-aminolevulinic acid-synthetase) and by inducing its degradation via heme oxygenase. These investigators have now shown that exogeneous injected heme can gain rapid access to a "free" or "unassigned" heme pool which serves as a precursor pool for cytochrome P-450. They succeeded in demonstrating this important new concept through an interesting experiment. It had been shown that a hypnotic-like compound, allylisopropylacetamide or AIA, turns the liver a green color when given in large doses to experimental animals. The compound responsible was isolated by another member of the Liver Center, Dr. Ortez de Montillano and shown to be an adduct of the heme portion of the drug-metabolizing enzyme (P-450) and the AIA. This causes a more rapid destruction of the enzyme; heme is therefore pulled from the heme pool of the cytoplasm to resynthesize the P-450 and in so doing allows new synthesis of heme by the liver by derepressing the rate-limiting step which takes place in the mitochondria. Dr. Correia and her group tritium-labelled the heme, injected it into an AIA-treated rat and showed (1) a much more rapid disappearance of heme from the plasma and (2) the appearance of the labelled heme in the cytochrome P-450. These observations provide a plausible rationale for the successful treatment of acute attacks of hepatic porphyria with intravenous hemin (heme chloride) infusions. These attacks can be precipitated by drugs that induce cytochrome P-450, such as barbiturates or AIA. Administration of heme may alleviate the attack by providing the heme to enter the "free" heme pool, thus preventing the need for an increased synthesis of heme by the liver cell, a process where an overshoot of the heme synthesis may be responsible for the porphyric attack. If infused hemin can also be incorporated rapidly into extrahepatic tissues such as the nervous system, it would also explain relief of the neurological symptoms of porphyric attacks. This experiment has not yet been done.

II. Contract-supported Activities

A multi-center clinical trial, the National Cooperative Gallstone Study, has been underway for some time. This study is evaluating the efficacy and long-term safety of chenodeoxycholic acid in the dissolution of cholesterol gallstones in a randomized, double-blind controlled trial. Over 1000 patients are currently in the study. The two-year period of medical treatment (375 mg/day or 750 mg/day chenodeoxycholic acid (CDCA), or placebo) will terminate in September 1980. Final analyses and publication of study results will then commence. Three groups of patients are emerging and will remain at completion of trial: (a) patients in whom gallstone dissolution is complete, (b) patients in whom gallstone dissolution is partial and (c) patients in whom no gallstone dissolution has occured. A new and very important study has been reviewed and initiated this year in which the group of patients with complete gallstone dissolution are being randomized into two groups receiving either low-dose (375 mg/day) chenodeoxycholic acid or placebo. The specific aim of this two-year study is to learn the recurrence rate of gallstone formation after complete dissolution and to evaluate the efficacy and further long-term safety of a maintenance dose of chenodeoxycholic acid in such patients. The results of this study should tell us whether it is feasible to consider the use of CDCA as prophylactic treatment in populations with high risk of forming gallstones. It should also tell us whether CDCA will be a drug which, like the antihypertensives, may have to be taken indefinitely, or whether intermittent therapy will protect against recurrence of gallstones once they have been dissolved.

III. Career Awards

In 1972, the Digestive Diseases and Nutrition Programs initiated a special 3-year career award (the Clinical Investigator Award) to provide additional research experience and guidance to promising young physicians beyond the normal 2-3 year post-doctoral research training. This additional experience was considered essential to provide the M.D. the expertise to become an independent investigator. We currently evaluated the success of our relatively limited experience with this program. Since the aim of the award was to produce an independent investigator, we analyzed the success of individuals who had completed the 3-year program in terms of their ability to obtain independent NIH research grants or RCDA support. Eighteen recipients have completed the CIA program between 1972 and 1975. From this group, the degree of success is as follows:

Research Applications

New (Applications funded/approved)	10/15
Renewal	8/8
Applications disapproved	1
Funded/submitted	18/24 = 75%

Research Career Applications

Applications funded/approved	4/5
Applications disapproved	2
Funded/submitted	4/7 = 57%

Training Grants

Applications funded/approved	2/2
Applications disapproved	0
Funded/submitted	2/2 = 100%

Total applications submitted	33
Total approved	30
Total funded	24

In conclusion, 18 individuals received a Clinical Investigator Award during 1972-1975. The end date for this analysis was chosen to allow completion of the 3-year period by 1978, thus allowing assessment of the degree of independence after completion. Thirty-three applications were received from these 18 investigators, of which all but 3 were approved. Twenty-four of thirty approved applications (80%) were funded, an average considerably better than for our total percentage funded during those years of 53, 32, 66 and 70%, for FY72, 73, 74, and 75, respectively.

ANNUAL REPORT (1978-1979)

BILIARY TRACT AND PANCREATIC DISEASES PROGRAM

A. Program Origin

The Biliary Tract and Pancreatic Diseases Program originated on January 4, 1979 as an offshoot of the ongoing Liver Diseases Program. Specifically, the applications and grants, including fellowships, careers, and research (but excluding all training grants and pre-existing research contracts), whose subject matter dealt with the biliary tract and its diseases (including bile salt and cholesterol metabolism, but excluding primary biliary cirrhosis and bile pigment and lipoprotein metabolism), the exocrine pancreas and its diseases, and the salivary glands and their diseases, were on that date transferred from the parent to the newly created program.

As of April 27, 1979, 2 individual postdoctoral fellowships, 7 career awards (6 research career development awards and 1 clinical investigator award), and 68 research grants were being supported by the Program. Approximately 66%, 29%, and 5% of the 77 activities under support were within the biliary tract, pancreas, and salivary gland areas, respectively.

B. Background Information

1. Diseases of the Biliary Tract:

(a) Cholesterol Gallstone Disease. It has been estimated that between 12 and 13 million people in the United States have cholesterol gallstones with all of their human and economic consequences. The advent of a medical treatment (chenodeoxycholic acid) that is effective in dissolving some stones is an advance of enormous importance. However, much more information is needed on the natural history of the long-term therapy. Further, it is generally held that prophylaxis is the ultimate objective, and for this we need a better understanding of the biophysical processes by which lipids are secreted in bile and nucleation occurs so that the pathogenesis of the disease state can be delineated.

(b) Pigment Gallstone Disease. Pigment gallstones, which constitute 24% of all gallstones, affect about 4 million people. Important advances have been made recently in the epidemiology and composition of these stones, but much remains to be done to elucidate the processes controlling pigment secretion, stone nidation and growth, and the role of biliary constituents in preventing precipitation. More basic knowledge is necessary before methods for dissolution and prevention can be devised.

(c) Cholestasis in Adults. Cholestasis, particularly that associated with the administration of a wide variety of medications, is an increasing problem. No effective therapeutic approaches are available, and the differential diagnosis is difficult. Progress will depend on elucidation of the basic mechanisms involved in bile secretion. Much has been learned recently about bile salt-dependent and -independent flow, but the molecular bases, while tantalizingly close, remain a mystery.

(d) Diseases of the Biliary Tract in the Newborn Infant. Treatment of most of these devastating diseases in infants and children is now based on surgical correction of the defect or liver transplantation. While these modalities need further development, the ultimate solution to the problem will be prevention - and this will require knowledge of the pathogenesis. Current knowledge is barely beyond the stage of classification.

2. Diseases of the Pancreas:

Among adults, the commonest diseases of the pancreas, other than cancer, are acute and chronic pancreatitis. Among children, cystic fibrosis is the commonest pancreatic disease, occurring in about 1 in 1,000 births. More than 100,000 persons are hospitalized each year in the U.S. for pancreatic diseases other than cancer. A far greater number who are not hospitalized suffer from pancreatic diseases. Alcoholism is by far the commonest cause of pancreatitis and gallbladder disease is the second most common cause, but in neither case do we understand how these conditions lead to pancreatitis. The enzymes of the pancreas digest food in the intestine. Normally these enzymes do not become active until they reach the intestine. In pancreatitis, the enzymes escape from the pancreatic cells into tissue spaces and become activated by unknown mechanisms. Research aims to find out why these abnormal events take place and how they can be prevented or reversed. The pancreas is a relatively remote organ, so only recently have diagnostic methods been developed which allow full display of its size, shape, and relations with surrounding organs. These new tools require evaluation. Much of our modern information about how cells manufacture proteins and package them for export to the exterior of the cell has been learned from studies in which the pancreatic cell served as a model. Palade won the Nobel prize for this work in 1974. It is important that this fundamental information on the biology of the pancreatic cell be pursued in such a way as to allow its application to the diagnosis, treatment, and prevention of pancreatic disease.

C. Progress Reports

Although a considerable amount of research activity, both basic and clinical, has been reported by the grantees during the past year, only a few examples will be cited to illustrate the progress that has been made in various areas of interest to the Program.

1. Biliary Tract:

(a) One of the puzzles of cholesterol gallstone formation has been that not everybody has gallstones. Almost the whole population, once every 24 hours, experiences conditions in gallbladder bile which should be conducive to gallstone growth, yet only about 10% of the population grow gallstones. Measurements at Carnegie-Mellon University of the rates of dissolution, nucleation, and growth of cholesterol monohydrate under a variety of conditions in model bile solutions containing bile salt, lecithin, cholesterol, and small electrolytes and in mixtures of human and model bile have elucidated some of the body's defenses against gallstone formation. So long as the daily concentration fluctuations in the gallbaldder remain within a normal range, the rate of nucleation is very slow, and the rate of dissolution at one extreme of the daily cycle is much faster than the rate of growth at the other extreme of the cycle. Even though the average daily concentration appears to favor growth, the interplay of dynamic processes inhibits it. Only when the upper and lower limits of the daily concentration variations are shifted towards abnormally high cholesterol concentrations do the nucleation and growth rates become rapid enough for net gallstone growth to occur. Cholesterol monohydrate growth has been found to be accelerated by the presence of calcium and magnesium but inhibited by potassium ions. This work has also produced information on the mechanisms of nucleation, growth, and dissolution which suggests that it will be more fruitful to search for ways to prevent or retard nucleation and growth than for ways to speed up dissolution.

(b) Grantees at the Hospital of the University of Pennsylvania compared gallstone disease in the United States and Japan and found that in Japan 19% of the patients have calcium bilirubinate stones, a type of stone associated with stasis and infection. This is rarely seen in the United States but can be identified using infrared spectroscopy. Nine percent of patients have black stones which have been found to develop exclusively in the gallbladder, since they do not recur after cholecystectomy. In contrast, bilirubinate stones comprise 90% of recurrent common duct and intrahepatic stones, both of which are rare in the United States. The remaining gallbladder stones contain predominantly cholesterol and resemble stones examined in the United States. A protein skeleton was identified for non-carbonate black pigment stones which was more prominent than in carbonate pigment stones. This suggests that abnormalities in protein secretion by the gallbladder may be involved in the etiology of non-carbonate stones. Patients with sickle cell disease were found to have stones identical in composition to patients with known hemolysis, suggesting that these two conditions have a similar etiology. Thus, it was hypothesized that pigment stones may form during an episode of transient hemolysis.

(c) Studies at the Peter Bent Brigham Hospital, Boston, MA have shown that the gallbladder is not an idle bystander in the formation of gallstones, but it reacts to the production of abnormal, cholesterol-rich bile by the liver with the outpouring of mucous, the slimey gel-like material produced by the lining of the gallbladder wall. It is thought that this mucous, which is probably produced as a defense mechanism by the gallbladder to protect against chemical injury, may, in fact, "turn the tables" on the gallbladder and act as a matrix within which cholesterol crystals nucleate and gallstones

grow.

(d) Work at Stanford University Medical Center involves studying the details of the mechanism by which dietary cholesterol reaches the liver. It has been known for a long time that lipids cannot circulate freely in the blood. They are incorporated into complex particles called lipoproteins. Dietary lipids are assimilated into a class of lipoproteins called chylomicrons. Only in the last few years has the metabolism of these particles become better understood. It is now known that these particles are metabolized in two steps. In the first step, they are depleted of specific lipids, the triglycerides, and a new particle is formed called a chylomicron remnant. This particle is rich in cholesterol. This particle is then selectively removed by the liver. The present work has been aimed at studying the mechanism of this process. Initially, using isolated livers, it was shown that the hepatic removal process is saturable and has a high affinity. More recently, by using partially purified plasma membranes from liver cells, a specific association between these particles and the membranes was identified. It appears that these particles bind to the membranes with a very high affinity. The process seems to be fairly specific for chylomicron remnants, since many of the other blood lipoprotein particles do not compete with remnants for sites on the membrane. Interference with binding caused by high density lipoproteins and unmetabolized chylomicrons was observed. These observations will form the basis for future studies which, it is hoped, will lead to an understanding of the precise mechanism by which cholesterol from the diet is ultimately incorporated into the liver. Understanding the mechanism of regulation of such a process may ultimately have utility for designing regimens to lower the blood lipid levels and may also have a role in understanding how much cholesterol is in the liver at any time and this may be important in understanding why people develop gallstones.

(e) Progressive liver disease terminating in cirrhosis and death is associated with early impairment and progressive deterioration of hepatic excretory function (cholestasis). Research at the New York Hospital - Cornell Medical Center is concerned with the pathophysiology of the initial events leading to progressive hepatic destruction and relates particularly to neonatal cholestasis and biliary atresia. Ten years ago it was found that certain bile acids (3β-hydroxy-5-cholenoic acid, 3α-hydroxy-5β-cholanoic acid), for which the name "monohydroxy bile acids" was intorduced, can completely stop bile flow (complete cholestasis). This effect is strikingly different from that of the predominant di- and trihydroxy bile acids normally found in bile which maintain bile flow (choleresis). It was demonstrated that the occurrence of cholestasis was dependent on the proportion of monohydroxy bile acids and led to the finding of 3β-hydroxy-5-cholenoic acid in amniotic fluid and meconium. It seemed, therefore, that a pathway for the synthesis of monohydroxy bile acids exists in fetal life. Using tracer techniques, it was possible to demonstrate a metabolic pathway from cholesterol, via 26-hydroxycholesterol, to mono, di, and trihydroxy bile acids. Some types of neonatal cholestasis can be attributable to an increased proportion of monohydroxy bile acids that must be excreted by the liver. It was also found that esterification (sulfation) of monohydroxy bile acids decreases their cholestatic effect and, therefore, the determinant of esterification and the effect of esterified bile acids on bile acid excretion and the

bile flow is now being studied. Currently, newer and more sensitive techniques to detect abnormalities in bile acid metabolism using tiny amounts (0.3 ml) of blood that can be obtained by heel stick are being developed. With these methods, it will be possible to identify infants at the earliest state of cholestasis and learn the natural course of the disease including the possible progression to irreversible atresia.

(f) Research at the University of Michigan has been aimed at understanding the mechanisms of cholesterol gallstone dissolution with emphasis upon the physical chemistry of gallstone dissolution kinetics and how such information may be utilized in improving existing methods and developing new methods for dissolving gallstones in patients. It was found that cholesterol dissolution in bile is much more rapid in more concentrated bile solutions, it is enhanced by electrolytes (more by Ca^{2+} than by Na^{+}), and it is retarded when the bile acid/lecithin ratio is decreased. The type of bile acid also influences the rate of dissolution, cholesterol dissolution being around 3 times faster in solutions of chenyl conjugates than in solutions of cholyl conjugates. Finally, it has been discovered that the addition of cationic amphipaths (amines and quaternary ammonium compounds) greatly enhances the cholesterol dissolution rates in bile by breaking the interfacial "barrier", and this finding has served as the basis for investigating a novel approach to accelerating gallstone dissolution.

2. Pancreas:

(a) Studies at the University of Colorado Medical Center on the structure and function of pancreatic enzymes have recently focussed on the precursors of two related enzymes, carboxypeptidase A and B, which feature in the final stages of the digestive breakdown of proteins into amino acids. The inactive precursors (molecular weight about 48,000) are split into enzymes (molecular weight 34,000) and smaller proteins (representing the weight balance), an unusual process because a large portion of the precursors is eliminated. To understand the physiological significance of this, the structures of the precursors are being determined. The precursor of carboxypeptidase A often occurs in various animals complexed with other precursors. In examining such a complex from hogs, the project identified and isolated for the first time a new and hitherto unrecognized enzyme precursor termed zymogen E. This major component of the secretion on activation yields an enzyme with properties reminiscent of those of pancreatic elastase. Elastase digests elastin, an important protein present in the walls of arteries and the bronchioles, and has been implicated in the etiology of emphysema. An understanding of the physiological significance of zymogen E awaits the outcome of further work.

(b) Inflammation is by far the most common disease of the pancreas in humans. Premature activation of zymogens and endogenous pancreatic proteinase inhibitors appear to be the two major factors in the pathogenesis of pancreatitis, inasmuch as the form, severity, and outcome of pancreatitis appear to be determined by the balance, at any particular point in time, between the amount of active proteinases formed in the organ and the amount of available proteinase inhibitors. The research ongoing in this connection at the University of Pittsburgh is two-pronged. On one hand, studies are directed at elucidating the alterations that arise in the pancreas and are responsible for

premature activation of zymogens. These studies are being carried out with a new experimental model of acute hemorrhagic pancreatitis developed in this laboratory. The disease is induced by dietary means in mice and has been shown to be determined by an overwhelming, premature activation of zymogens. The most recent findings indicate that this activation is caused by cathepsin B_1, a lysosomal enzyme and an activator of trypsinogen to trypsin. These findings, if corroborated, may open a new approach for the therapy of pancreatitis, since potent inhibitors of cathepsin B_1, devoid of side effects, are available. Their efficacy is presently being tested in the laboratory. On the other hand, studies are directed at investigating whether the amount of proteinase inhibitors present in mammalian pancreas is under genetic control, as is the case of plasma α_1-antitrypsin. Studies in this connection have shown, so far, that significant differences exist between male and female and between different strains of inbred rats.

(c) Preliminary studies ar Cornell University Medical College indicate that histamine stimulates pancreatic secretion and potentiates the effect of other pancreatic stimulants. However, the nature of this control and the physiological relationship of histamine to known pancreatic stimulants (i.e., CCK-P_N, acetylcholine, chymodenin) remain to be explored.

A different series of investigations carried out at the same laboratory has provided evidence that pancreatic digestive enzymes, once secreted by the pancreas into the lumen of the intestine, can be absorbed intact from the gut, transported back to the pancreas, and resecreted by the pancreas. Recent related findings have suggested that pancreatic enzymes in the intestine elicit chloride secretion in the ileum. Such secretion could help maintain fluid in the intestine needed for digestion. The extent, utility, and control of this enteropancreatic circulation of digestive proteins and the characteristics of the molecular mechanisms mediating the necessary transcellular movement of intact proteins await further investigation.

3. Salivary Glands:

(a) Studies at the Veterans Administration Hospital, Hines, Illinois have led to the discovery of substantial quantities of a large glucagon-like material in the submaxillary glands of man and animals. Because of the pivotal role of glucagon, thought by some to influence the course of diabetes meleitus in man, better understnading of the role of extrapancreatic glucagon seems highly desirable. During 18 months of study, it has been possible to demonstrate de novo synthesis of this large glucagon-like material in the salivary glands and to reconfirm its biologic activity in stimulating release of glucose from the liver. Although of much higher molecular weight than pancreatic glucagon, this material is capable of binding competitively to hepatocyte membrane glucagon receptor sites in a fashion comparable to that of pancreatic glucagon. Unexpected discoveries during the course of these studies have demonstrated the presence of immunoreactive insulin in the parotid glands of man and animals and the presence of immunoreactive insulin in saliva which changes in a physiologic fashion during such tests as glucose administration. Recently, large amounts of immunoreactive glucagon and insulin have been discovered in pancreatic exocrine secretions, as well. Studies are currently under way to further characterize these materials and to determine what roles they may play in nutrient absorption and carbohydrate

metabolism, in particular. Finally, the presence of glucagon in the exocrine submaxillary glands has proved to be androgen-dependent and the significance of this observation will be further pursued. Recent observations from several centers have noted the complete absence of microvascular disease in prepubertal juvenile-onset diabetic children with the development of such disease following puberty in poorly controlled diabetic youngsters. What role the sex steroids may play in such a development will have to be carefully investigated.

NIAMDD ANNUAL REPORT FY 79

NUTRITION PROGRAM - DDN

The Nutrition Program, NIAMDD-DDN, budget for FY 1979 was $13.300 million to carry out its primary responsibility of supporting research, manpower development and training in basic and clinical nutrition. In addition, $2.243 million of diabetes-related nutrition research (25 research grants, including the obesity center) funded by the Diabetes Program, was administered by the Nutrition Program making a total of $15.518 million. This will include three Clinical Nutrition Research Units (CNRS) to be funded from FY 79 funds.

The Nutrition Program will fund 133 research grants including 3 program projects and 3 CNRU's in FY 79. However, the number of research grants active during the year will be 154, or 179 including diabetes related grants administered. This represents an increase of 11 grants funded (including 3 CNRU's) or an increase of 15 grants active during FY 79 as compared with FY 78.

Approximately 43% or 39 of 90 research grants approved by the National Advisory Council were funded. Other programs within the Institute support nutrition related research, especially Digestive Diseases, Hematology, Orthopedics, Endocrinology and Metabolism Programs; the total NIAMDD supported nutrition research and training is estimated to be 20% of that administered by NIH.

Priorities

The Nutrition Program has continued to stress the following nutrition priorities: study of human nutritional requirements and factors that influence these requirements; use of new technologies in the study of the function of nutrients; studies on iron deficiency; research levels and metabolic roles of trace minerals; basic studies on dietary fiber; research on the interaction of nutrition, immune competence, and infection; increased support for obesity research with the aim toward obesity prevention; and studies on supportive nutrition for hospitalized patients, through Clinical Nutrition Research Units.

Workshops

NIAMDD supported or cosponsored the following workshops or conferences during FY 79. These were designed to identify gaps in research and to acquaint investigators with priority research needs, especially those requiring a multidisciplinary approach:

1 Concensus workshop on "Surgical Treatment of Morbid Obesity", December 4-5, 1978, Bethesda, MD (through Nutrition Coordinating Committee).

2. International Conference on "Effects of Alterations of Dietary Patterns and Food Habits on Health with Special Reference to Critical

Analysis of Comparative Epidemiological Studies of Different Populations", December 5-6, 1978, Osaka, Japan (cosponsored by the US and Japan Malnutrition Panels, US-Japan Cooperative Medical Science Program).

3. Workshop on "Methodologies for Human Population Studies in Nutrition Related to Health", July 24-25, 1979, Bethesda, MD (cosponsored by the US and Japan Malnutrition Panels, US-Japan Cooperative Medical Science Program.

4. Fourth Workshop on Vitamin D,, February 18-22, 1979, Berlin, West Germany (cosponsored by Nutrition Program and Urology and Kidney Diseases Program, NIAMDD).

5. Conference on Micronutrient Interaction, February 20-22, 1980, New York City (funded through FIC in FY 79).

6. Workshop on "Nutritional Support of Patients: Research Directions for the 1980's", September 17-18, 1979, Washington, DC (through the Nutrition Coordinating Committee).

Training

At present there is a real need to train manpower in both basic and clinical nutrition research. Efforts are being made to expand training support. Under the National Research Service Award program support of 9 institutional training grants was continued, with 21 predoctoral, and 24 postdoctoral or a total of 45 trainees. It is hoped that funds will allow the funding of one new training grant before the end of FY 79.

Also under the National Research Service Award program, 3 new individual postdoctoral research fellowships were awarded, making a total of 13 funded and 18 active during FY 79.

Nutrition research manpower development is also enhanced by research career development awards. Nine K04's in nutrition were funded. A total of 12 RCDA's were active in FY 79, including 1 clinical investigator (K08), 1 academic career (K07) and 1 career award (K06).

US-Japan Program

The NIAMDD Nutrition Program administered the US-Japan Malnutrtion Panel, Cooperative Medical Science Program. The US Malnutrition Panel has continued to support research relating to the nutritional health problems of Southeast Asian people. The redefined objectives are as follows:

1. The influence of environmental and host factors on nutritional requirements.

2. Health significance and methods of preventing iron deficiency.

3. Interaction of nutrition, immune competence and infection.

4. The effects of nutrition on physical and mental development, behavior, physical capability and work performance.

5. The health consequences of different dietary patterns and food intake.

These emphasis areas were detailed in the NIH Guide For Grants and Contracts. The panel supported two workshops in FY 79 in collaboration with the Japanese Panel. One was held in Osaka, Japan on "The Effects of Alterations of Dietary Patterns and Food Habits on Health with Special Reference to Critical Analysis of Comparative Epidemiological Studies of Different Populations", December 5-6, 1978. The other is being held at NIH July 24-25, 1979 on "Methodologies for Human Population Studies on Nutrition Related to Health".

NIAMDD will fund seven grants totaling $535,000 in FY 79 as a part of this program.

Recommended Dietary Allowances

The Food and Nutrition Board, NRC, NAS, was funded by contract to complete the ninth revision of the RDA's. This will be available to NIAMDD by mid-July and should be published late in 1979. This latest revision of the RDA's will include provisional values for fluorine, manganese, copper, selenium, chromium, molybdenum, sodium and potassium.

Also a special report was requested concerning 1) an evaluation of the scientific basic from which the "Dietary Goals for the United States", published by Senate Select Committee on Nutrition and Human Needs, were derived and 2) gaps in current knowledge which should receive priority attention. This report is expected to be completed in June 1979.

Research Supported by Grants

1. Fat Soluble Vitamins

Wirtz (West Virginia University) has developed a radioimmunoassay for retinol in serum, able to reliably measure as little as 5 nanograms and requiring no more that 1 ml. of blood. By appropriate extraction protocol, it will be possible to separate retinoic acid from retinol-retinal and follow small changes in tissues.

Lamb found that cytochrome p-450 mediated hydroxylations are depressed in stringently vitamin A deficient rats. This has significance in connection with the molecular function of vitamin A and in the detoxication of potential carcinogens.

Johnson (Oklahoma Medical Research Foundation) has obtained results which indicate that for growth and development of the fetus, both retinol and retinoic acid are required. It is known that in the rat retinoic acid alone will maintain the adult animal except for vision and reproduction.

Extensive studies are underway by Dr. Goodman and others at Columbia University on several aspects of the metabolism and transport of vitamins A and D. One aspect involves the elucidation of the cellular and molecular mechanisms involved in the regulation of retinal binding protein production in the liver. A retinoic acid binding protein has been identified in rat testes which is distinct from that found in the cytosol for retinol.

The project concerned with vitamin D relates to the protein, called DBP, which is responsible for the transport of vitamin D and its metabolites in human plasma. They are characterizing DBP, exploring its interaction with various vitamin D metabolites and studying the effects of diseases on plasma levels. Serum levels of DBP were found to be moderately decreased in patients with chronic hepatitis and decreased in patients with early acute hepatitis or cirrhosis.

Work on mechanism of action of vitamin A by Wolf (MIT), suggests that the compound formed from UDP-galactose and retinyl phosphate in tracheal microsomal systems is retinyl phosphate galactose. During a vitamin A deficiency, the level of membrane bound UDPgalactose: glycoprotein galactosyl transferase was diminished by 50%.

DeLuca's group (University of Wisconsin) has isolated and identified two vitamin A compounds from the intestinal mucosa of rats given 450 mcg of ^{3}H labeled retinoic acid. The 5, 8-oxyretinoic acid has been isolated in pure form. Also, they have isolated the 5, 6-epoxyretionic acid from the intestinal mucosa of vitamin A deficient rats given tritiated retinoic acid. The 5, 6- epoxy-retinoic acid is a potent form of vitamin A in the reversal of keratinization of epithelial cultures, in the suppression of ornithine carboxylase induced by phorbolesters applied to skin of mice and in the growth of vitamin A deficient rats. It should be noted that 5, 8-oxyretinoic acid is easily produced by acid treatment of a 5, 6-epoxide.

A grantee (Norman) and others at the University of California at Riverside has recently detected by radioimmunoassay a vitamin D-dependent calcium binding protein in rachitic bone, which differs in molecular weight from the previously identified calcium binding protein in the intestine. It is concentrated principally in the spongy and cartilage plate regions of the bone, and may have important clinical implications by virtue of its probable role in bone formation and resorption.

Dr. Normans group has developed a radioimmunoassay for calcium binding protein with a sensitivity of one nanogram. They also have obtained evidence that one of the vitamin D_3 metabolites, 24,25-dihydroxy vitamin

D_3, is required biologically for reproduction in the chicken. This was not previously recognized. This research group also has tested active vitamin D_3 metabolites in clinical studies in normal subjects and uremic patients.

A major advance has been made by the Wisconsin Group (DeLuca) and others in the understanding of the future metabolism of 1, 25-$(OH)_2D_3$. The existence of an intestinal 25-OH-D_3-24-hydroylase has been demonstrated. The product has been isolated and identified as 1, 24 R, 23-trihydroxy vitamin D_3. Thus an extrarenal site of 24-hydroxylation of the D-vitamins has been found.

Hoekstra (University of Wisconsin) in studies on the metabolic role of vitamin E and selenium in peroxidation, has measured the ethane in the expired air of animals as an index of lipid peroxidation. Vitamin E was found to protect more effectively than selenium.

Studies were conducted by Corwin, Boston University, on the effects of dietary vitamin E and fatty acids on mitogenesis and antibody synthesis. Vitamin E acts as a mitogen itself. Using cell fractionation technique it was demonstrated that it is a beta cell mitogen and a T cell mitogen. Vitamin E is an antioxidant in some biological systems and it has been shown to decrease prostaglandin synthesis from PUFA, presumably by inhibiting a necessary peroxidatin step.

Progress in the area of vitamin K by Suttie (Wisconsin) has centered around the purification of the vitamin K dependent carboxylase, clarification of the role of oxygen in the preparation, and the characterization of the reaction products.

A stable vitamin K-dependent carboxylase has been obtained from rat liver microsomes.

The oxygen involvement in the reaction has been studied by the generation of various possible active oxygen species as superoxide, hydroxy radical, singlet oxygen, or by their inhibition. These data tend to support a free radical type of mechanism for the reaction. Studies of the product of the carboxylase and the single substrate Phe-Lew-Glu-Lew- have indicated that the major product is carboxylated only in the first Glu residue.

2. Water-Soluble Vitamins

Studies on the absorption of simple and conjugated folate in alcholic patients is being systematically conducted by Halsted (University of California, Davis). This work is defining the role of altered intestinal hydrolysis in folate malabsorption of alcoholism and will assess the role of recent drinking and liver disease in folate assimilation. Another investigator (Waxman, Mt. Sinai) is studying the tissue distribution, role and mechanism of folic acid binding protein in transport, delivery and intercellular localization of folate.

Jacobson (Scripps Clinic) has recently made some exciting observations, using scanning electron microscopy, concerning the transport of cobalamins into mamalian cells. Membrane receptors for transcobalamine II-cobalamin complexes appear to be located in microvilli.

In metabolic studies on vitamin B6, Fonda (University of Louisville Foundation) has purified, characterized and developed an assay for a pyridoxal kinase. This should greatly facilitate the study of the regulation and interconversion of the vitamin B6 forms found in tissues.

McCormick (Cornell) has conducted detailed studies on the metabolism of riboflavin containing enzymes and flavoproteins. delineating the nature of flavin binding and ascertaining the catalytically functional amino acid residue of certain flavoproteins. The biochemical properties of hepatic mitochondria from riboflavin-deficient rats, monitored during recovery showed that riboflavin was incorporated into four mitochondrial flavo-proteins with covalent bound flavin coenzyme. Alterations in cytochromes, biosynthesis of succinate dehydrogenase, monamine oxidase, sarcosine dehydrogenase and an unknown flavoprotein were observed.

3. Proteins and Amino Acids

Considerable progress has been made by Walser and coworkers (Johns Hopkins University) in developing effective therapeutic agents for the treatment of congential hyperammonemia and portal systemic encephalopathy, using new compounds composed of branched-chain keto acids and amino acids. These new compounds are considerably more palatable and acceptable to the patient (neutral and sweet in taste) than are the amino acid or keto analogues (usually bitter, metalic taste). These compounds have real promise in permitting the improvement of protein nutrition and delaying dialysis or transplantation in end stage renal failure.

A very timely finding by Eisenstein (SUNY Down State Medical Center) is that high protein diets resulted in the increased production of glucagon and insulin from isolated islets of Langerhan. More recent work has shown that a high level of a single essential amino acid is needed for isolated cultured pancreas cells to produce these hormones. This may prove important if pancreatic transplants become a more feasible means of preventing diabetes.

Flatt (University of Massachusetts) in studies on the amino acid and protein needs of patients has shown, that increased administration of amino acids during parenteral nutrition at fixed carbohydrate caloric intakes results in a retention of more nitrogen and higher rates of net protein utilization. Restoration of body cell mass during parenteral nutrition can be accelerated by increasing the dose of amino acids in relation to dextrose.

Young (MIT) has iniated some novel approaches, including alpha-15 N-lysine and 1-^{13}C-leucine as tracers, to assess body protein metabolism adaptation to alterations in intake and adequacy of protein and energy in healthy subjects.

Adibi and others at Montefiore Hospital, Pittsburgh has studied the mechanism of intestinal assimilation of dipeptides and tripeptides containing glycine and leucine; studies of gastric secretion by amino acids and peptides; and effects of peptide on gastric emptying. This work suggests that a tripeptide diet might be superior to a elemental diet for patients with digestive and absorptive disorders.

Wurtman (MIT) has found that tyrosine administration significantly reduces blood pressure in spontaneously-hypertensive rats or DOCA-salt treated hypertensive rats.

Studies at University of Wisconsin (Harper) have continued on dietary factors that influence the metabolism and transport of specific amino acids into the brain, and the behavioral consequences which may result, including changes in food intake. Studies have shown that the level and balance of dietary neutral amino acids does effect the concentrations in brain tissue. Since many amino acids are precursors of neurotransmitters, changes in the concentration of these may influence behavior.

Munro and others at MIT have initiated studies to provide information on the nutritional regulation of protein synthesis in the liver and placenta. The synthesis of ferritin has been explored in relation to iron administration. These researchers have demonstrated that the pool of free messenger RNA for ferritin is mobilized into polyribosomes when iron is given to rats.

Benevenga, University of Wisconsin, has shown that the transaminative pathway of metabolism of methionine degradation is active in the human liver. This finding suggests a new approach to the therapy of homocystinuria, by converting homocysteine to methionine with supplemental betaine. Already two homocystinuric patients have been treated with betaine for over 6 months with marked clinical benefit.

4. Trace Elements and Newly Recognized Dietary Minerals

Work by Suttie, University of Wisconsin, was designed to determine the nutritional and metabolic consequences of the ingestion of fluoride by animals (or the inclusion of fluoride in the growth media of cultured cells). Particular efforts were directed toward an assessment of the possible effects of fluoride ingestion in erythrocyte ATPase concentration and a more complete understanding of the effects of fluoride on cultured cell metabolism. (The consumption of an increased intake of fluoride by a large segment of the US population is ample justification for such studies).

Prasad (Wayne State University) observed decreases in zinc concentrations of plasma, erythrocytes, leukocytes and urine of four male volunteers who received restricted intakes of zinc for several weeks under metabolic conditions. Changes in the activities of zinc dependent enzymes in plasma, such as alkaline phosphatase and ribonuclease were also related to zinc status. This study provides a basis for developing diagnostic criteria for zinc deficiency in humans.

Cousins (Rutgers University) has found that metallothionein mRNA is associated with free polysomes, rather than membrane bound polysomes, indicating that metallothionein is an intracellular protein. His studies lend stong support to the role of metallothionein in zinc metabolism in the liver and intestine and that the metabolism of zinc is highly regulated. Other studies by this investigator show that zinc metabolism is a dynamic process at the cellular level. Cadmium accumulation and its effect on zinc metabolism in liver cells has been studied.

Owen (Mayo Clinic) has initiated studies on the abnormal copper metabolism in the Bedlington terrier, in hopes that this may provide an understanding of the mechanism of Wilson disease in man.

An improved method of measuring magnesium absorption using stable ^{26}mg as a tracer has been developed by Schwartz (Cornell). Even though the method requires fecal samples within 3-4 days and urine samples within 2-24 hours use of ^{26}mg (the stable isotope) significantly expands the scope of investigation of magnesium absorption in man beyond that possible with the short lived radioisotope ^{28}Mg alone.

Crosby (Scripps Clinic and Research Foundation) has continued studies on monocyte ferritin in hemochromatosis. Dr. Crosby's work on the red blood cell ghost delivery system of iron chelators shows great promise for application to the treatment and prevention of the lethal accumulation of iron that occurs in thalassemia and related disorders. Entrapped in red blood cell ghost permits iron chelators (desferrioramine) to reach the cells where iron is stored.

Studies by Carlisle, UCLA, has found that a dietary deficiency of silicon resulted in abnormal skull formation and a reduction of collagen content. This effect of selenium inadequacy on bone formation was found to be independent of vitamin D. Because of such a profound effect of silicon on collagen formation, a better understanding of its role should be important in preventing and treating connective tissue disorders.

An improved method of measuring serum and urine chromium levels in normal and diabetic patients has been developed by Vanderlinde (Hahnemann Medical College and Hospital).

Longitudinal studies on zinc and copper nutriture during pregnancy and lactation, in low birth weight and breast-fed infants and in acrodermatitis

enteropathica are underway at the University of Colorado (Hambidge). Eighty preschool children will be enrolled in a zinc supplementation study during the coming year.

Kopito (MIT) is doing zinc balance studies on adult males in the attempt to establish the dietary requirement.

5. Dietary Fiber

Oace, (University of California at Berkeley) has standardized model systems for evaluating the effects of dietary fiber on intestinal transit time, fecal enzymology and the bioavailability of micronutrients. A vitamin B_{12} depleting effect of pectin has been established firmly. Measurement of fecal enzymes, alpha-and-beta-galactosidase, beta-glucosidase, alpha-and-beta-xylosidase and beta-glucuronidase in rats, revealed highly specific effects for particular fiber treatments.

Macy (University of California, Davis) in studies on the influence of dietary fiber on rat intestinal bacteria, revealed that more cellulose digestors are present in the gut, as well as a different, more complex bacterial population, when rats are fed a high-fiber diet. Products of degradation of "dietary fiber" thus may be involved in any benefical health effects.

Studies by Anderson (V.A. Hospital, Lexington) designed to determine if generous amounts of starch and plant fibers have any advantageous over those conventionally used to treat individuals with diabetes mellitus. The results obtained thus far suggest that high-carbohydrate, high-fiber diets may be the dietary therapy of choice for patients with maturity onset type diabetes mellitus and may have distinct benefit for patients with hyperlipidemia. Long term studies showed that diets containing 50 grams of plant "fiber" per day are well tolerated and show no adverse nutritional effects.

6. Fats and Fatty Acids

Privett is attempting to elucidate the metabolism of dietary fatty acids in relation to lipid composition of mammalian tissues with emphasis on the role of essential fatty acids (EFA) in these reactions.

Trans-trans-linoleate blocks the interconvension of linoleate to arachidonate and it inhibits the conversion of linoleic to arachidonic acids. A monenoic fatty acid is produced in abnormal concentrations upon feeding diets containing trans fatty acids.

In studies by McCay (Oklahoma Medical Research Foundation) on the relationships between dietary antioxidants, lipid peroxidation and free radical events associated with the metabolism of carcinogenic substances in liver cells a spin-trapped radical has been demonstrated in the metabolism of halothane by liver microsomers. The functions of vitamin E and the selenoenzyme,

gluthione peroxidase, are being studies with respect to the mechanism of inhibition of lipid peroxidation in biological membranes.

Similar studies by Tappel (University of California, Davis) have involved the use of a new non-invasive technique which measures pentane gas production as an indicator of lipid peroxidation. These studies have shown 1) that dietary vitamin E or other antioxidants inhibit pentane production, and 2) that the recommended dietary level of selenium protects to about one-half that obtained with vitamin E.

Oscai (University of Illinois) has found that the newborn rat can be programmed by controlling food consumption in early life for a premanently lower or higher voluntary food intake. They also have found that lipoprotein lipase can act as a mobilizing enzyme capable of hydrolyzing endogenous triacylglycerols. The evidence suggests also that this enzyme may be involved in the regulation of tissue triglycerides in the heart.

Romsos (Michigan State University) has observed that polyunsaturated fatty acids specifically inhibit fatty acid synthesis in the liver (rat). The in vivo rates of fatty acid synthesis closely approximated the activity of acetyl CoA carboxylase and fatty acid synthetase, thus supporting the suggestion that the level of activity of fatty acid synthetase determines the maximum rate of fatty acid synthesis from carbohydrates in meal-fed rats. He also has observed a reduced activity of Na^+, K+ ATPase activity in muscle of genetically obese mice. This may account for the reduced energy expenditure in these animals and hence their potential for obesity.

In studies of glycerol metabolism, Hirsch and others have not shown any defect in glycerol metabolism in obese subjects, beyond the fact that larger masses of adipose tussue are associated with a greater release and turnover of glycerol. During the past year they have been able to show that administering glycerol to rats either subcutaneously or orally will decrease food intake and body weight. Glycerol has a distinctly greater effect than an equivalent amount of glucose. This affect occurred by reduction of meal frequency during the might portion of the the feeding cycle.

7. Obesity

During the past year the Obesity Center at St. Lukes Hospital (Van Itallie) has developed a Weight Control Unit to provide a comprehensive approach to calorie management for better health.

Studies with the recently perfected universal eating monitor have progressed effectively in measuring differences in eating patterns of obese and lean individuals. Individuals ate fewer calories when a highly palatable diet was modified to contain less energy, suggesting the low-calorie

analogues are effective in promoting weight loss. Added fiber has been used with success in certain foods.

Studies on "protein-sparing" low calorie diets for weight loss have shown that a hypocaloric diet consisting only of protein does not spare body nitrogen better that an equicaloric mixture of protein and carbohydrate.

Evidence that the signal that terminates voluntary eating is dependent on a substance transmitted through the blood stream in the rat has been obtained by Koopmans (Columbia University) by using a supernumery, transplanted stomach and animals with two-way and one-way crossed intestines. The signal can arise from either the stomach or small intestine after food is injected.

Hirsch (Rockefeller University) found that early undernutrition in rats has no permanent effect on fat cells in terms of size regulation, but does restrain their production and perhaps, the production of adipocyte precursor cells as well. A relationship between insulin level and fat cell size was observed which is independent of cell number, degree of obesity or amount of food consumed. Even in severe genetic obesity in the rat, the nature of the diet was found to be an important factor in the expression of the disorder.

Debons, in studies on the mechanism of action of gold thioglucose, has found that a small non-necrotizing dose of gold thioglucose does not affect serotonin levels in the ventromedical hypothalamus, but did produce an increase in ketone body formation in mice.

Work by Woods at the University of Washington suggests that the insulin level in the cerebral spinal fluid may be a signal that informs the brain as to present adiposity, thereby influencing food intake. Manipulations of CSF insulin in baboons did affect food consumption.

Kanarek (Tufts) and coworkers have found the monosodium glutamate administration to neonatal rodents leads to obesity even through the treated animals actually eat less than the controls. It is obvious from their studies that small differences in activity levels as well as daily calorie intake are important in the development of obesity.

Houpt and others (Cornell) in studies on preabsorptive intestinal satiety control of food intake, concluded that duodenal osmoconcentrations to the degree that it exceeds body fluid toxicity contributes to the immediate satiety seen during a meal.

Powley (Yale) is mapping the preganglionic parasympathetic system controlling pancreatic secretion, gastrointestinal secretion and motility as related to feeding and body weight control. This should have major experimental and clinical significance.

Dietary effects on metabolism (high-carbohydrate vs high-fat) were studied by Kaplan (Rutgers State University) using the 3 to 6 week-old ob/ob mice and paired controls. The reduced consumption of oxygen in the ob/ob mouse is the first indicator of this genetic obese condition. Diets high in fat were found to produce higher oxygen consumption than high carbohydrate diets in such animals. Also total lipid and percent lipid were decreased by high fat feeding in ob/ob and non ob/ob males. These observations suggest that the type of diet fed early in life may have profound consequences on energy metabolism and resultant body composition in later life.

Work at Washington University (Fisher) is directed toward developing and refining behavioral procedures for the encouragement of clinically significant weight losses among individuals sufficiently over weight to of medical concern. A self-control manual has been developed which has been effective.

Efforts to improve the methodology for measuring body composition are under way by Buskirk (Pennsylvania State University). Excretion of 3-methylhistidine excretion as a measure of muscle mass is being studied along with the use of tritiated water.

Keesey (University of Wisconsin) has studied the metabolism adjustments of both normal and lateral hypothalamically-lesioned animals to experimentally induced changes in their body weight. Reductions in resting metabolic rate and heat increment were observed in underweight animals.

Kidney Disease and Urology Program

The rapid growth of the Kidney Disease and Urology (KD&U) Program, coupled with the integration of the Artificial Kidney/Chronic Uremia Contract Program into the KD&U Program in January 1979, has required an administrative reorganization, in which three sub-programs were designated.

Renal Physiology and Pathophysiology Program: Has responsibility for the scientific administration of all research grants, program projects, contracts and manpower development programs that not only relate to normal structure and function studies of the kidney, including metabolism, transport and fluid electrolyte dynamics, but also to those studies that are in the area of pathogenesis of renal disease.

Urology, including Urolithiasis Program: Oversees the scientific administration of all research grants, program projects, urolithiasis SCOR grants, contracts and manpower development programs that relate to the diseases of the lower urinary tract, including accessory sex organs, and urolithiasis (upper and lower urinary tract).

Chronic Renal Disease Program: Is responsible for the scientific administration of all research grants, program projects, contracts and manpower development programs that relate to pathophysiology of chronic renal failure, kidney transplantation and preservation, the evaluation of therapies for treatment of end-stage renal disease patients and critical issues related to the maintenance of chronic uremia patients.

This reorganization will be completed by the end of the current fiscal year and the total delineation of the grants for each section should be concluded prior to the October 1979 Council.

In terms of dollars administered by the Kidney Disease and Urology Program during FY 1979, the support for investigator-initiated research grants, ROIs (236), program projects (7), centers (5), contracts (62), Clinical Investigator Awards (7) and Research Career Development Awards (17) exceeded $31.8 M, while the support for National Research Service Awards, both institutional (30) and individual (32) was $2.102 M.

Training.

One new institutional National Research Service Award (T32) was funded in FY 1979, bringing the total to 30 active training programs supported by the KD&U Program, which provide stipends for 82 postdoctoral and 6 predoctoral trainees. Added to the 27 active individual National Research Service Awards (F32s) were 5 new awards, increasing the total to 32 fellowships supported with FY 1979 funds. Four new Research Career Development Awards were made, bringing the total to 17 and the Clinical Investigator Award Program has been increased to 7, by the addition of 5 new commitments.

Conferences.

During FY 1979, the KD&U Program provided partial support for several major international meetings, including: (1) Joint U.S. - U.S.S.R. Symposium on Biological Membranes held in Kiev, U.S.S.R., October 1-14, 1978; (2) Symposium on Prostatic Cell and Organ Culture, November 27-29, 1978, Lake Placid, New York; (3) Fourth Workshop on Vitamin D, February 18-22, 1979, Berlin, West Germany; (4) 1979 International Prostaglandin Conference, May 27 - June 1, 1979, Washington, DC; (5) Conference on Anion Transport in Epithelia, Isolated Cells and Cell Membranes, New York City, June 18-21, 1979; (6) 4th International Workshop on Phosphate, June 22-24, 1979, Strasbourg, France. In addition, a Conference on Immune Mechanisms in Renal Disease was jointly sponsored with the National Institute of Allergy and Infectious Diseases at the NIH during the period of October 2-4, 1978. A monograph covering the proceedings of the meeting is being prepared for distribution in late 1979.

RENAL PHYSIOLOGY AND PATHOPHYSIOLOGY PROGRAM

Analgesic Nephropathy.

A two year epidemiological study of analgesic use related to renal disease will be completed in early 1980, which should lend some credence to the hypothesis that analgesic abuse is a cause of end-stage interstitial nephritis. The 120 cases for the study were selected from among persons on renal dialysis in the Greater Delaware Valley who will be compared to age-sex-race matched controls that have been selected at random from among persons attending a Health Evaluation Center. The research, if an association is found, should provide important information relative to the etiology of at least some forms of end-stage renal disease and may possibly provide important implications for prevention following other studies.

Nephrotic Syndrome.

During a period of eight years, a network of ten U.S. hopsitals and medical centers engaged in a collaborative study, in which 250 patients were enrolled and studied. Several pilot studies were included in this effort, but the major work involved previously untreated adult nephrotics with several types of biopsy-proved histology. Perhaps the major finding to date was the discovery that a two month course of alternate-day prednisone therapy significantly slowed the rate of deterioration of glomerular filtration rate (from -10% to -2% per year) in patients with membranous nephropathy. In two completely comparable groups, 11 placebo-treated patients reached renal failure, contrasted with 2 prednisone-treated patients during the same period of observation. It is also of note that even in one small study group of 72 patients, the onset of chronic renal failure was postponed for some nine patients in the treated group. Another unexpected finding involved adult nephrotics with minimal change disease. Although prednisone is customarily given for this condition, it appears, within the limits of the number of

patients studied and the duration of follow-up, that there is no long-term benefit to these patients as a result of steroid therapy.

Glomerulonephritis (GN).

An observation of a nonbasement membrane glomerular antigen being involved in spontaneous GN in rabbits by an investigator at Scripps Clinic and Research Foundation has led to an evaluation of human serum and renal eluates for similar reactivity. A look at several hundred serum samples over the past several years has revealed occasional samples which appear to react in a granular, nonbasement membrane distribution along the glomerular capillary wall when studied by indirect immunofluorescence. In addition, at least one renal eluate from an individual with GN has had similar reactivity, strongly suggesting that occasionally nonbasement membrane glomerular antigens may be involved in human GN. Intensive studies are now underway in the laboratory to develop more sensitive and reproducible assays for detecting this reactivity and for identifying the antigen involved in both the rabbit and the human.

The importance and the need for expansion of studies to further the understanding of the immunopathogenesis of human renal disease, especially GN, is enforced by recent reports in the literature. For example, among the first sixteen thousand renal transplants reported to the American College of Surgeons' Registry, more than half were performed in patients who had some form of diagnosed chronic glomerulonephritis. In a review of incidence in chronic renal failure in Great Britain, 38 per million per year in the age group 5-60 years was reported and of these, 36% were considered to have chronic glomerulonephritis. Clearly, the medical, social and economic impact of glomerular diseases throughout the world is enormous.

Pathogenesis of Diabetic Nephropathy.

The thrust of a recently awarded new proposal is to establish whether the increased binding of albumin to basement membrane of diabetic patients is the consequence of an alteration of albumin or of a primary alteration of basement membrane induced by the diabetic condition. The initial results of this investigator's research suggest that there is a characteristic increased albumin localization within diabetic renal basement membrane. To obtain more definitive information to confirm his early results, there is a special resource tissue bank of 80 frozen diabetic kidneys.

Developmental Renal Physiology.

The use of currently available sophisticated methodology, such as micropuncture and microperfusion, to study developmental renal physiology is relatively new and the number of trained investigators in this field is small. As a result, knowledge about maturation of renal transport mechanisms is being developed. Two pediatric nephrologists recently were awarded grant support, one to examine the passive movement of fluid and solutes across the peritubular membrane of the proximal tubule of the developing kidney and the other, to study phosphate

transport in the neonate, and factors which promote the high rates of phosphate absorption in the developing guinea pig. These studies should help to determine the factors that regulate nephron function and initiate the rapid changes in function that take place after birth.

Kidney Cell Culture.

New and useful data concerning function and growth of the mammalian kidney epithelial cell line MDCK (an epithelial cell derived from dog kidney), is anticipated from the laboratory of a recently funded investigator at the UCSD. These cells display several functional and anatomical properties of normal kidney tubule cells including brush borders, formation of tight junctions, and catalyis of unidirectional sodium transport. In pertinent preliminary work, the investigator reports that these cells possess high activities of Na-K-ATPase, the amiloride sensitive sodium channel, ecto-leucine aminopeptidase and ecto-alkaline phosphatase. Adenylate cyclase activity was found to be responsive to vasopressin, oxytocin, prostaglandins E_1 and E_2, and glucagon. In addition, he has succeeded in developing a hormonally defined serum-free growth medium for the MDCK cells and the plans for isolation of variant clones resistant to amiloride should provide important information concerning the expression and control of kidney specific functions.

Mathematical Modeling.

In recent years, refined mathematical models have been established in order to explain how water and solute transport in the mammalian kidney results in concentration of urine. The differential equations which have been devised are non-linear and their solutions must satisfy boundary conditions which differ in the various regions of the organ. An accomplished mathematician at SUNY at Stonybrook, who is experienced in problems of numerical analysis, has been collaborating with an intramural NIH group for the past five years, the latter doing the modeling while this investigator works on numerical methods. The role for this modeling effort continues to provide important contributions to the understanding of renal function by relating membrane concentration profiles and input-output data. Furthermore, this effort is developing efficient computer algorithms for handling the equations that arise in modeling various aspects of renal physiology, including particularly, the countercurrent problem in the medulla of the kidney.

UROLOGY, INCLUDING UROLITHIASIS PROGRAM

The five Specialized Centers of Research (SCORs) in Urolithiasis are nearing the end of a second year of a five year commitment. The annual SCOR meeting is proving to be useful in disseminating information and keeping each group abreast of advances that are being made. The second meeting is scheduled for October 4-5, 1979 and will be hosted by the Mayo group in Rochester.

The University of Texas, Southwestern group has developed a protocol for an ambulatory technique for diagnosis of urolithiasis. Subsequently, this group has computerized data from 150 consecutive patients with renal stones, who underwent the ambulatory diagnostic evaluation. With data retrieval and analysis, it will be possible to formulate minimal diagnostic criteria for different causes of stones, to assess important physiological inter-relationships and to define the clinical presentation of each disorder. This data base will be useful in future clinical trials.

One goal of the Mayo Group during the initial years of the award was to identify a cohort of the Rochester population whose medical history had presented any evidence of renal colic or symptomatic renal stones. It has been established that virtually complete case ascertainment is possible in this community, which is especially health oriented, probably due to the Mayo Clinic's presence in Rochester for over 60 years. Initial evaluation of the records of these cases was geared to elucidation of the age specific and age adjusted incidence of renal lithiasis in the community during the 1950-1974 period. Plans include the evaluation of recurrence rates, seasonal variations and related incidence by sex. Of 672 patients who were identified, 468 were males and 206 were females. The age distribution of the incidence episodes showed the peak incidence was between 30 and 60 in men but was constant from age 20 to 70 in females. The overall incidence rate was 109/100,000 for men and 36/100,000 for women. Female rates were remarkably stable over the study, but there was a 50% rise in the incidence rate from 1950 to 1974 from 80/100,000 to 124/100,000 in men. This appeared to be exclusively in the men from ages 35 to 70. The recurrence rate for stones in patients having their initial stone was also calculated and it was revealed that there is a 16% chance of recurrence in the first year for men and 12% for women. Thereafter, the recurrence rates were constant for at least 15 years, being 3.7% for men and 2% for women. These rates did not appear to change over the years as did the incidence rates. Furthermore, although the number of individuals with a second recurrence was too small for calculation of precise rates, the rates were approximately twice as high as for the initial recurrence. A seasonable variation could not be demonstrated.

Antibacterial Defense Mechanisms.

Experiments underway at Jefferson Medical College suggest that the surface mucin of the rabbit bladder mucosa is an important antibacterial defense mechanism which functions by preventing bacterial adherence to the bladder wall, thereby facilitating the removal of bacteria during the voiding process. Although the relationship of this layer in the human bladder to infection has yet to be determined, this investigator purports that the surface mucin may be regarded as an important antibacterial defense of the bladder. Consequently, if a deficiency or abnormality in this layer is seen in chronic or recurrent infections, further studies may well lead to the understanding and better treatment of urinary tract infections.

In related studies at the University of Wisconsin, an investigator is studying factors within the urinary tract collecting system that are thought to allow bacterial adherence to mucosal surfaces. For example, studies are underway to

determine if intra-vesical iodine complexes, or bacterial antigens in the bladder or bacterial antigens in the vagina are most protective. Preliminary data indicate that dilute organic iodine complexes were the most effective anti-adherence agents with acceptable morbidity. Other studies are planned to determine genetic differences by quantitating urothelial adherence of C-57/black, BALB/c and other inbred mouse strains.

Spinal Cord Injury.

Important studies are underway at Yale University on neurophysiology of urine storage and micturition in normal animals and in animals with spinal cord transections, simulating spinal cord injuries. These studies are designed to provide further information on the control of lower urinary function after spinal cord injury, with particular reference to studies of electrical stimulation techniques for the inhibition of tonic responses of the urinary bladder to filling, and for the periodic production of coordinated detrusor emptying. Although the initial work is being carried out on cats, the investigator will also do some of the studies on primates, which should be helpful in the application of the results to the urologic management of spinal cord injuries in man.

Benign Prostatic Hyperplasia.

Two major grants continue to explore the mechanisms underlying Benign Prostatic Hyperplasia (BPH). One investigator is providing a better understanding of the molecular mechanisms involved in the actions of androgens and their antagonists in the accessory genital organs, primarily the prostate. The other group, supported by a program project, is heavily involved in studies of the comparison of experimentally induced canine BPH with that of the spontaneous disease occurring in pure-bred beagle dogs of known age. Their recent studies of spontaneous prostatic hyperplasia in the beagle has confirmed that this is a disease that increases in frequency with age, although the onset may occur as early as 2-3 years of age. Two distinct types of spontaneous hyperplasia have been recognized; glandular hyperplasia is characterized by an elaborate projection of a hypertrophic epithelium into the lumina of enlarged alveoli. The histological appearance of glandular hyperplasia may vary considerably, from an early stage of the disease to one which is markedly abnormal. Cystic hyperplasia is characterized by cuboidal, but hyperplastic epithelium, with the formation of large cysts, and an increase in the ratio of stromal to epithelial cells. This group suggests that glandular hyperplasia probably represents an early stage in the development of the disease, preceding the appearance of cystic hyperplasia.

CHRONIC RENAL DISEASE PROGRAM

During FY 1979, the activities of the former Artificial Kidney/Chronic Uremia Program were integrated into the Kidney Disease and Urology Program. In the

next fiscal year, it is anticipated that support of research in chronic renal disease by the Institute will shift toward more emphasis on investigator-initiated research projects with contracts being reserved for selected, appropriate projects.

Developments in Kidney Transplantation and Artificial Kidneys.

The NIAMDD is one of several Institutes of the National Institutes of Health supporting research aimed at improving transplantation procedures for kidneys and other organs. Transplantation is a desirable means to treat patients with end-stage renal disease, but the frequent rejection of transplanted organs makes this surgical approach far less consistent in the results obtained than treatment with artificial kidneys. Overcoming the problem of rejection of grafted kidneys continues to be one of the most serious obstacles to a predictable outcome in the use of transplantation, although there has been progress in this area in recent years. In general, patients who receive well-matched organs from closely related donors have the best chance of success among recipients of transplants and of a consequent return to a more normal existence.

The main technique used in treating end-stage renal disease for more than 15 years has been maintenance hemodialysis, which is now used in about 44,000 patients in the U.S. It has successfully maintained life in patients whose outlook was otherwise hopeless. Although transplantation offers an attractive alternative when compatible donor organs are available, hemodialysis has remained the single most important treatment modality, and in the case of end-stage polycystic kidney disease is often considered the treatment of choice.

Long experience with maintenance hemodialysis has demonstrated that many difficulties can arise during the course of treatment. These problems may be associated with the uremic state, regardless of present treatment, or may arise from the treatment itself. Specific examples are: neuropathy (primarily impaired sensation or muscle strength in arms or legs) in some patients, osteodystrophy (decreased mineral content of the bones), sexual dysfunction and anemia. In addition, the well-being of patients on hemodialysis is impaired by occasional muscle cramps, hypotension and post-dialysis fatigue, especially when large amounts of fluid have been removed during dialysis treatments, which are given three times each week. The mortality rate among maintenance dialysis patients is about 9% a year regardless of sex or age; this figure must be viewed in contrast with a certain 100% mortality for patients with non-functioning kidneys if dialysis or transplantation were not available for treatment.

Managing Transplant Patients with Viral Infections.

Cytomegalovirus (CMV) infections are known to cause significant secondary illness in renal transplant patients and those with bone marrow transplants as well as other disorders associated with suppressed natural immunity. Grantees of the NIAMDD at the University of Minnesota, after conducting prospective studies, have suggested that the finding of leukopenia (reduced number of white

cells in the blood) is significant in early recognition of CMV infections in renal transplant patients. The study has revealed that onset of such infections is usually in the second month after kidney transplantation, that it is often accompanied by a reduced number of leukocytes, and that early recognition of such infections may be very helpful to the transplant surgeon in the management of the patient's immunosuppression regimen. The investigators also say that if differences among CMV strains can be detected by serologic techniques, it will be possible to trace a single virus type from patient to patient, from donor to transplant recipient, or from patient to hospital personnel. The researchers have now undertaken a CMV infection screening program among the members of the transplantation service at the University of Minnesota, and they anticipate that this surveillance study over a four year period will provide answers to important epidemiologic questions.

Effect of Saralasin in Hypertensive Patients on Chronic Hemodialysis

Appropriate therapeutic management of hypertension in patients on hemodialysis requires a fuller understanding of the underlying mechanism. Blood volume overload may be responsible, in which case adequate ultrafiltration should normalize the blood pressure. This procedure does not apply, however, in the case of hypertension mediated by the renin-angiotensin system which affects the systemic arteriolar bed. Since measurement of plasma renin activity has failed to differentiate these two patient categories, an NIAMDD grantee at the University of Texas Health Science Center at San Antonio undertook tests to determine the capacity of saralasin, an angiotensin II antagonist, to lower the blood pressure in hypertensive patients.

Patients were divided into two groups on the basis of their blood pressure response to saralasin. Responders categorized as angiotensin II-dependent were expected to benefit from subsequent nephrectomy. Results obtained by the investigators indicate that renin-angiotensin-mediated hypertension in patients on chronic hemodialysis may be corrected by saralasin, which antagonizes angiotensin II, and by bilateral nephrectomy, which removes the source of abnormally high levels of renin. The increased plasma renin activity in responders suggests antagonism by saralasin of the inhibitory feedback loop of angiotensin with respect to renin release. Lower plasma renin activity of nonresponders, however, may reflect either absence of angiotensin or an inoperative feedback loop, thereby excluding mediation of blood pressure by the renin-angiotensin system. The researchers conclude that saralasin is a safe drug for detection of renin-angiotensin-mediated hypertension in patients on chronic hemodialysis, and is useful in predicting amelioration of this disorder by bilateral nephrectomy.

Continuous Ambulatory Peritoneal Dialysis (CAPD)

The technique known as peritoneal dialysis utilizes the lining of the abdominal cavity (the peritoneum) as the membrane through which uremic waste products are filtered. Blood is not removed from the body as in hemodialysis. Instead, a sterile wash solution is repeatedly introduced into the abdominal cavity and withdrawn from it through a small tube that is permanently implanted in the

abdomen. Each time, the wash solution is left in the abdomen for about 20 minutes during which toxic substances are removed through the peritoneal membrane by osmosis.

A dramatic improvement in this technique is currently under investigation by contractors of the Institute at the University of Texas, Austin Diagnostic Clinic and the University of Missouri. With NIAMDD sponsorship, these researchers are studying the technique of continuous ambulatory peritoneal dialysis (CAPD) to determine the long-term effects of what seems to be a simple and potentially inexpensive form of therapy which frees the patient from having to come to a dialysis center three times weekly for his dialysis treatment.

Patients in CAPD therapy maintain about two quarts of dialysis solution in their peritoneal cavity 24 hours per day, except for short periods required for drainage of dialysis fluid and installation of fresh solutions several times during their waking hours. The enthusiastic acceptance of this treatment method by patients and the potential economies, make it a promising new approach to maintenance treatment of end-stage renal disease. Further research on CAPD is urgently needed in order to bring the concept to broader clinical application and to provide a better, less restricted quality of life to the many patients afflicted with chronic renal failure. From today's knowledge, it appears that peritoneal dialysis is likely to become far more important as a mode of maintenance treatment than it has been until now.

Metabolic Effects of Exercise Training in Hemodialysis Patients.

The Program has also been involved in supporting a feasibility study in physical exercise as a means to diminish abnormalities encountered in hemodialysis patients which may be of significant importance in the pathogenesis of their excessively high cardiovascular-related mortality rate. At Washington University, St. Louis, contractors are conducting this study.

It is known that patients with chronic renal failure who receive long-term hemodialysis develop a broad spectrum of medical complications, including hypertension, atherosclerotic cardiovascular disease, abnormal lipoprotein metabolism, impaired glucose homeostasis, hyperinsulinism, hyperparathyroidism with abnormal bone and mineral metabolism, and anemia. Despite impressive advances in the care of these patients and in hemodialysis technology, quality of life remains unsatisfactory and long-term survival is often limited by the accelerated development of atherosclerotic cardiovascular disease, the mechanism for which is not clear.

Based on the fact that exercise is widely used in the rehabilitation of patients with ischemic heart disease, and endurance exercise training improves cardiovascular hemodynamics in normal individuals, this study was undertaken for the purpose of determining: (1) if hemodialysis patients could safely participate in an exercise training program, (2) whether exercise training would improve endocrine, metabolic, and/or cardiovascular abnormalities, and (3) if this increase in physical activity would be reflected in an improvement in the psychosocial functioning of hemodialysis patients.

Seven subjects with chronic renal failure requiring hemodialysis for 4-5 hours three times weekly were studied; mean duration of hemodialysis was 49 ± 33 months. Subjects were selected by the following criteria: (1) plasma HDL-C concentration at least one standard deviation below the normal range (45 ± 10 mg/dl) and elevated plasma triglyceride concentration for age and sex, (2) absence of medical problems contraindicate participation in an exercise training program, (3) stable medication regimen, and (4) motivation to participate. All subjects were receiving aluminum hydroxide for hyperphosphatemia, all were anemic, and two were treated with antihypertensive medication. Prior to the initiation of exercise training, all subjects underwent: (1) examination by a cardiologist, (2) resting electrocardiogram and echocardiogram, (3) symptom limited Bruce graded exercise stress test (GXT), and (4) maximal oxygen consumption (VO_2 max) test. Patient initial GXT duration was very low and their maximal oxygen uptake, an index of work capacity, was lower than that expected for age and sex matched normal subjects (37 ml/kg/min).

Results show that there has been an increase in exercise tolerance with increased caloric expenditure. In the subjects exercising for the longest period of time, there has been a reduction in both plasma triglyceride and cholesterol levels and an increase in plasma HDL-C concentration. Concomitantly, in male subjects a striking increase in both hematocrit and hemoglobin was observed. Two of the subjects have been able to reduce the antihypertensive medications; in two patients, serum phosphate levels decreased following training. Furthermore, all participants expressed that as a result of the exercise training they felt physically and emotionally better.

It is apparent from these preliminary results that exercise training may cause major improvement in hemodialysis patients in terms of cardiovascular function, blood pressure reduction, serum lipoprotein changes, reduced serum phosphate levels, rise in hematocrit, and psychologic improvement which have occurred over a short interval. All these findings suggest that exercise training may have great therapeutic potential in the dialysis population. Further longitudinal studies on a larger number of dialysis patients are necessary to confirm these potential benefits of exercise training and to ascertain the mechanisms by which they occur.

Hematology Program

The Hematology Program of the National Institute of Arthritis, Metabolism and Digestive Diseases supports extramural investigation into basic mechanisms of normal blood cell function and disease pathogenesis, and ranges through development of treatment modalities, to clinical application and evaluation of treatment of relevant diseases. This research increases both fundamental and applied knowledge about blood. Further, the availability of blood and blood cells facilitates study of membrane phenomena, protein structure and function, etc., to obtain information broadly applicable across the spectrum of medical problems, in areas such as chronic renal insufficiency, transplantation, and malignancy.

Major hematologic categories supported are:

1. anemias of genetic origin, particularly the hemoglobinopathies, with emphasis on regulation of hemoglobin and hemoglobin synthesis (e.g., Cooley's anemia, sickle cell anemia).

2. metabolic disorders related to iron transport and storage (hemosiderosis), porphyrin metabolism (porphyrias), and enzymatic defects (e.g., red cell glucose-6-phosphate dehydrogenase deficiency).

3. nutritional anemias, especially those involving iron, folic acid, and vitamin B_{12}.

4. disorders of blood cell production, such as those encountered in systemic and/or metabolic disorders. Examples are the anemia of chronic renal failure, aplastic anemia, and polycythemia.

5. immunology of hematopoietic tissue transplantation and of autoimmune hematologic disease.

The Hematology Program budget for the support of extramural research and training represents slightly more than 6.2 percent of the total NIAMDD extramural budget for Fiscal Year 1979, as compared to 6.1 percent the previous year, showing that the steady relative decline since FY 1974 has been at least temporarily altered. The increase for the program from FY 1978 to FY 1979 was about 22.6%, which will provide a significantly better funding of grants.

Anemias of Genetic Origin

The genetic regulation of hemoglobin synthesis continues as one of the most active areas of investigation supported by this program. Aberrant hemoglobin synthesis represents a suitable model for the study of genetic disease in general, and the system could provide information directly applicable to the treatment, cure, or even prevention of hematologic disorders such as sickle cell anemia and β thalassemia. Much of the nucleotide sequence of several normal human globin mRNAs has been determined, by means of sequence

analysis of human globin cDNA-plasmid DNA. The broadened use of recombinant DNA techniques will bring more complete information on normal and abnormal components of the genetic regulatory process, and eventually should result in a complete characterization of the process.

Progress in understanding the molecular basis for thalassemia has been made in a number of areas. In β^0 thalassemia, where there is an absence of β chain synthesis, some workers have found the presence of substantial amounts of β messenger RNA. In one group of patients, half had an absence of β mRNA, but in the other half there was 5-15% as much β-like mRNA as α mRNA. This β-like mRNA is functionless in cell-free protein synthesizing systems. It appears that the β-like mRNA in β^0 thalassemia may be relatively unstable and subject to more rapid degradation in vivo than normal β mRNA. The specific nature of this instability needs to be examined. An investigator supported by the program has reported success in isolating the major part of the DNA concerned with human fetal globin chain synthesis. His first comparisons of these human fetal genes with their adult counterparts have shown that the sequences that intervene within the coding regions are in the same locations in both sets of genes. This establishes that control of the switch from fetal to adult hemoglobin cannot be the result of the fetal genes lacking these intervening sequences. It remains to be seen whether the specific base sequences of any parts of the intervening sequences are important in this context.

Prenatal diagnosis of thalassemia, by fetal blood analysis, has been applied successfully to samples containing as little as 5% fetal blood, and is being improved. These techniques are being used in many countries where thalassemia is prevalent, including Italy, Greece, and England. It may be worthwhile to consider a clinical trial designed to standardize the many techniques that are being practiced.

The only currently available treatment for thalassemia is chronic transfusion, which ultimately leads to fatal iron storage disease. The contract program whose goal has been to develop new and clinically more effective iron chelating drugs to decrease iron overload has screened more than 120 candidate drugs. One promising group of compounds may be the esters of ethylenediamine N,N^1-bis (2-hydroxyphenyl) acetic acid , which have the potential for oral use, and which have higher efficacy than the parent compound. The latter has been tested for subchronic toxicity, with relatively good results. Studies are being planned for further work on this and other potential chelators.

Metabolic Disorders

Disorders of iron metabolism, whether characterized by a deficiency, excess, or inability to utilize this essential metal, are among the most common and widespread of human maladies. Effective therapy is usually available for simple iron-deficiency anemia, but refractory anemias, acute iron poisoning, chronic iron overload, and sideroblastic anemias, pose therapeutic challenges.

The amount of research supported on iron storage, transport, and metabolism disorders continues to expand, especially as more basic scientists enter the

field. A mini-symposium on coordination chemistry in medical science, with emphasis on iron chelators is planned for the September, 1979 annual meeting of the American Chemical Society. The NIAMDD effort to develop new iron chelators for clinical use has seeded new interest in this area. A second trend is the increased interaction of clinical and basic investigators, to a large extent fostered by the biennial international conferences on proteins of iron metabolism, sponsored in part by the NIAMDD, and the series of annual, but informal, meetings of Iron Clubs, being held on the east and west coasts.

In vivo studies by one investigator showed that iron deficient rats absorbed increased amounts of lead, and iron overloaded rats absorbed less lead than control animals. Intraluminal doses of lead also decreased the absorption of test doses of lead from the small intestine in a dose-related manner, while lead-laden animals and the addition of lead to test doses of iron had no significant effect on iron absorption. The investigator postulates that this occurred because the two metals competed for similar binding sites on intestinal mucosal proteins important in the absorptive process. In vivo, both lead and iron bind to a heat-stable mucosal protein of a molecular weight of 370,000, and certain other recently-described physico-chemical properties. Further, the addition of iron to test doses of lead markedly diminished the amount of lead bound to this protein, while lead had little effect upon the amount of iron bound to the mucosal protein. The differential affinity of this protein for iron and lead could explain the high incidence of lead poisoning in iron deficient children.

Significant progress has been made in elucidating the detailed subunit composition of isoferritins from normal and malignant tissues from humans and horses. These multiple forms of ferritin share extensive sequence homologies among their subunits, but each contain unique peptides. This suggests that the subunits represent products of distinct but related genes. Also, the regulation of synthesis of both subunits is under separate metabolic control, as shown by preferential induction of one subunit by iron in normal cells, a control which may not operate in malignant cells.

Nutritional Anemias

Nutrition surveys continue to demonstrate the prevalence of iron-deficiency anemia among young children, adolescents, and women of menstrual age. Because milk is consumed widely, it is a logical vehicle in which to deliver supplemental iron and copper to improve hematological status, particularly during periods of rapid growth. The nutritional value of ferric and cupric chelates of nitrilotriacetate and lactobionate has been examined, and they appear to be safe and efficacious food additives. Supplementation of milk using these agents may correct dietary deficiencies of both elements while being free of negative odor and flavor problems of other agents previously tried.

Folate and vitamin B_{12} deficiencies or metabolic defects are significant causes of anemia. A dual radioisotopic assay for the simultaneous measurement of folate and vitamin B_{12} has been developed, which gives results comparable to those of separate radioassays in half the time and manpower. The deoxyuridine suppression test using transformed lymphocytes has been developed as

a routine clinical test for past folate or B_{12} deficiency in treated subjects, and for current masked folate deficiency in iron-deficiency. Neither are detectable without this test.

Disorders of Blood Cell Production

Hypoplastic anemias and primary polycythemias often occur because some multipotential hematopoietic stem cells inappropriately develop into mature red blood cells. To determine the reasons for this, it is necessary to identify and characterize undifferentiated stem cells committed to later formation of erythrocytes. Recently-developed culture methods have allowed the necessary detection and quantitation of erythroid stem cells both in humans and in animals. Erythroid precursor cells have been induced to make colonies of erythroblasts in semi-solid cultures of plasma clots and methyl cellulose, during incubation. Erythroid colonies derived from late stage erythroid precursor cells, called "colony forming units - erythroid" (CFU-e), contain up to 40 erythroblasts, while the more primitive erythroid stem cells are referred to as "burst forming units-erythroid"(BFU-e), and give rise to colonies containing hundreds of erythroblasts.

Important studies are under way using these new systems. For example, the subpopulations of lymphocytes and other mononuclear cells capable of influencing in vitro erythroid colony formation are being studied with implications for diseases of marrow failure, such as pure red cell aplasia and aplastic anemia. Marrow and peripheral blood culutures for erythroid pregenitors are being established both before and after removal of selected populations of lymphocytes to determine the effect of such removal on marrow growth. In 15-20% of patients, no erythroid colony formation was observed in unfractionated mononuclear cells, until the T-lymphocytes were removed. In such patients, normal colony formation was demonstrated following removal of T-cells and the readdition of autologous T-lymphocytes abrogated colony growth. These data suggest that some patients have cell-mediated interactions which may result in clinical marrow failure.

Chronic renal disease is always accompanied by anemia of major clinical signifcance. While anemia is multifactorial, its primary cause is a decreased rate of red cell production relative to the degree of anemia. Decreased functional renal mass results in diminished production of erythropoietin. To some extent, hyperphosphatemia and acidosis may affect red blood cell production. The degree to which erythropoiesis is suppressed varies from patient to patient, and is correlated partly with the severity of uremia. The uremic rat is a useful model for the study of effects and mechanism of action of potential therapeutic agents, such as anabolic and androgenic steroids, which significantly increase hematocrits of uremic patients.

Extrarenal sites of erythropoietin production probably exist in the liver and perhaps also in the spleen. Extrarenal sites, as well as those in the kidney, respond to hypoxia and to administered agents, but are only weakly stimulated by androgen therapy. Because it is unlikely that exogenous erythropoietin will be available in sufficient amounts for treatment of anemia of uremia, it is necessary to search for conditions under which extrarenal erythropoietin

production can be enhanced. One investigator supported by this program found that the extrarenal erythropoietin production of anephric rats is enhanced 2-4 days after partial hepotectomy and after feeding carbon tetrachloride, during a time when liver cell regeneration is occurring at maximum rate. Work is planned to define the cells responsible, and the conditions under which their activity is greatest. In addition, an interesting effect of certain vasodilators in increasing extrarenal erythropoietin is being pursued.

Immunology

The objective of much of the immunologic research supported is to determine the underlying mechanisms for the specific association of HLA-antigens with certain diseases. For example, HLA-B27 is specifically related to Reiter's disease. Since post-shigellosis Reiter's disease is apparently triggered by a particular strain of Shigella, one study is focussing on antigenic differences between species. An enzyme-linked immunosorbent assay for the B27 antigen is under development, and when completed, would be used to screen shigellosis patients for B27 positivity, and to test the association of bacterial antigens with the B27 antigen. Such a test has been developed for β_2-microglobulin, and work on the B27 test is well underway.

A reliable simple test has been developed to detect granulocyte antigen and has potential for widespread use. Prior to this development, complete definition of granulocyte antigen systems has been very difficult. The new test method should be applicable to clinical cases for defining possible autoimmune and isoimmune mechanisms of granulocytopenia. In immunized patients, granulocyte transfusion may be of no value if antibody seriously affects granulocyte function and survival. A high incidence of granulocyte cytotoxins has been reported recently in renal transplant patients who rejected the graft. The importance of granulocyte cytotoxins in transfusion therapy and tranfusion reactions is being examined.

Evaluation of the Hematology Program of NIAMDD in Relation to Research Needs

The evaluation anticipated last year is under way, with Albert Einstein College of Medicine in New York City as the contractor (Dr. Ernst Jaffe Principal Investigator). The study has the cooperation and active support of the other institutes with blood programs, notably NHLBI and NCI. Working committees are beginning their review of the various hematologic areas of research, to be submitted to the Coordinating Committee early in 1980. The final report is expected by 1981. The hematologic areas being reviewed are: 1) Hematopoiesis, Normal, 2) Hematopoiesis, Abnormal, 3) Lymphopoiesis, Normal, 4) Lymphopoiesis, Abnormal, 5) Granulocytes and Monocytes, Circulating, 6) Erythrocytes, Circulating, 7) Nutrition and Malnutrition, 8) Platelets and Coagulation Factors, 9) Immunohematology, 10) Plasma Proteins.

Manpower Development

Fiscal Year 1981 National Research Service Award funds will not permit the Program to initiate new individual and institutional awards. The number of individual NRSAs will fall from 15 to 9, while several excellent individual and institutional applications have been recommended for approval, but cannot be funded.

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS
AND SCIENTIFIC COMMUNICATION

SCIENTIFIC COMMUNICATIONS SECTION

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The Scientific Communications Section has the responsibility for general programming and planning of communication activities concerned with the various scientific disciplines that are charged to NIAMDD. The present activities can be generally grouped into four main categories:

1. Current awareness literature services;
2. Retrospective literature studies;
3. Conference and workshops; and
4. Other aspects of communication, such as thesarus development, production of motion pictures, etc.

Current awareness literature services are provided by the Institute in several specialized areas. At present, there are four publications with hierarchically arranged listings of literature citations and one publication which includes abstracts. GASTROENTEROLOGY ABSTRACTS AND CITATIONS, now in its eleventh volume, is intended to provide selected abstracts and literature citations in all aspects of gastroenterology, biochemical or clinical. Four of the publications, ENDOCRINOLOGY INDEX (twelfth volume), DIABETES LITERATURE INDEX (fourteenth volume), INDEX OF DERMATOLOGY (eleventh volume), and KIDNEY DISEASE AND NEPHROLOGY INDEX (fifth volume) contain citations to current worldwide literature that have been included by the National Library of Medicine in their Medical Literature Analysis and Retrieval System (MEDLARS) as well as citations gleaned from other sources of biomedical literature.

To produce these structured, hierarchically-arranged indexes that function as indepth current-awareness tools for comprehensive and easy review of particular subjects, we have cooperated with the National Library of Medicine, and in one case, also with the University of Minnesota and the University of Rochester. The standarized terminology used in MEDLARS makes these publications invaluable for retrospective literature searching and invaluable to those who are interested in these highly specific areas.

For each publication, outstanding scientists working in the field covered by the publications have been assembled as editorial advisors to insure continuation of the high quality of these publications.

Conferences, symposia, and workshops sponsored by the NIAMDD are planned and organized by the Scientific Communications Section. In addition to the organization of the conference with the conference chairman (including physical arrangement as well as program planning), the publication of the proceedings also falls under the auspices of the Scientific Communications Section.

This year ten conferences, workshops, and a congress, sponsored completely or jointly with other Institutes or organizations, were held. Conferences and a congress included the Twelfth Annual Contractors' Conference of the Artificial Kidney-Chronic Uremia Program, Behavioral and Psychosocial Issues in Diabetes Conference, Consensus Development Conference on Surgical Treatment of Morbid Obesity, Conference on Immune Mechanisms in Renal Disease, and the Second International Congress on Nutrition in Renal Disease. Workshops included the Can Diet Be Dialysis Sparing? Workshop, The Potential of Exercise in Maintenance Dialysis Workshop, Directions for Research in Osteogenesis Imperfecta

Workshop, Hemoperfusion for Kidney and Liver Supports and Detoxification Workshop, and Psoriasis Dialysis Workshop. Less extensive support, usually along with other Institutes, was contributed to conferences on transmethylation, mediation of effector functions by antibodies, molecular and cell biology of polyamines, prostatic cell and organ culture, clinical aspects of cyclic nucleotides, biological recognition and assembly, Cooley's anemia, prostaglandins, frontiers of science and the liver, computed tomography for tissue densitometry, peptides, gastrointestinal motility, and aplastic anemia.

Proceedings and conference summaries included ELEVENTH ANNUAL CONTRACTORS' CONFERENCE PROCEEDINGS, PROCEEDINGS OF AN INTERNATIONAL NUTRITION CONFERENCE ON BEHAVIORAL EFFECTS OF ENERGY AND PROTEIN DEFICITS, PROCEEDINGS OF THE NATIONAL CONFERENCE ON CLINICAL TRIALS METHODOLOGY, FOURTH INTERNATIONAL CONFERENCE ON BONE MEASUREMENT PROCEEDINGS, and the SUMMARY OF WORKSHOP ON ARTIFICIAL DEVICES FOR THE CONTROL OF BLOOD GLUCOSE.

National Library of Medicine's on-line MEDLARS (Medical Literature Analysis and Retrieval System) is available to NIAMDD staff through NIAMDD's call number. The Scientific Communications Section is responsible for coordinating this use and serves as a reference source for all of NIAMDD staff (Extramural, Intramural, and Administrative).

In addition, numerous other activities that bear on scientist-to-scientist communication as it affects the Institute are dealt with frequently. Areas where further emphasis possibly should be placed are continually in review.

Project Title:	DIABETES LITERATURE INDEX
Contract Number:	NO1-AM-7-0663
Amount:	$133,875
Contractor:	University of Minnesota
Project Officer:	Mrs. Billie B. Mackey
Man Years:	Total: .40 Professional: .15 Other: .25

Project Description

The DIABETES LITERATURE INDEX, now in its fourteenth volume, is a current-awareness publication produced monthly, and a yearly cumulative issue also is produced. This bibliography of diabetes-related literature was brought about through the cooperative efforts of the NIAMDD, the National Library of Medicine, the American Diabetes Association and three universities, (University of Minnesota, University of Rochester, and Case Western Reserve University), as well as many individual scientists.

The National Library of Medicine's MEDLARS data base, referencing all of the current biomedical literature citations to both journals and monographs, is used to retrieve all diabetes-related bibliographic citations monthly. Besides the primarily diabetes-related literature that is included, approximately one third of the citations of interest to diabetologists and others working in the diabetes field are carefully selected and culled from the peripheral literature. The DIABETES LITERATURE INDEX, until 1971, consisted of a keyword in title index and an author index and was prepared from a computer printout. Since 1971, a magnetic tape of these indexes is prepared at the University of Minnesota and is used to generate copy for offset printing by the U.S. Government Printing Office on the Linotron. Three formats are included in the publication, a hierarchical subject index of core diabetes literature, an abbreviated keyword in title index of all diabetes literature, and an author index including index terms with the citations (listed under the first author). The citations are grouped under a preconceived hierarchical structure with related subjects grouped together making it easier for users to scan their particular interests in one section.

The DIABETES LITERATURE INDEX is published by the Scientific Communications Section, NIAMDD monthly and is available to National Institutes of Health grantees or contractors with interests in diabetes, government agencies with responsibilities in this area, and to medical school libraries. It is for sale by the Superintendent of Documents, U.S. Government Printing Office for a fee. The free mailing list was circularized to insure currency.

Project Title: ENDOCRINOLOGY INDEX

Professional Personnel: Mrs. Billie Mackey

Man Years:

Total:	.5
Professional:	.2
Others:	.3

Project Description

The ENDOCRINOLOGY INDEX, now in its twelfth year of publication, is a current-awareness journal produced bimonthly. Approximately 4,000 citations of the current world literature in journals and monographs of interest to those in the field of endocrinology are included in each issue. This bibliography is produced with the aid of the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS), by the Scientific Communications Section, NIAMDD. The format of the publication consists of citations grouped in eight categories (pituitary, thyroid, etc.), and each category has a preconceived hierarchical organization of subject headings. Index terms, assigned by the indexer, after perusing the original article, appear in the author section, and author and subject indices appear in each issue.

From other sources, citations are selectively added including monographs and articles of potential interest to neuroendocrinologists chosen from six publications of the Brain Information Service. Ongoing work with the Medical Subject Headings Section of the National Library of Medicine for inclusion of endocrine-related vocabulary in the computerized vocabulary is conducted.

ENDOCRINOLOGY INDEX is available to National Institutes of Health grantees and contractors with interests in the field of endocrinology, government agencies with responsibilities in the field, and medical school libraries. It is for sale by the Superintendent of Documents, Government Printing Office for a fee. The free mailing list was circularized to insure currency.

Project Title:	GASTROENTEROLOGY ABSTRACTS AND CITATIONS
Contract Number:	NO1-AM-4-2204
Amount:	$153,895
Contractor:	Franklin Institute
Project Officer:	Mrs. Billie B. Mackey
Other Professional Personnel:	Dr. Andrew Greenhill Dr. Gladys Hirschman Dr. Antonia Novello Mrs. Elsie Yuen
Man Years:	Total: .30 Professional: .15 Other: .15

Project Description

GASTROENTEROLOGY ABSTRACTS AND CITATIONS, a monthly current-awareness journal for gastroenterologists, has been published by NIAMDD since January 1966. Each issue, prepared by contract, contains approximately 350 abstracts and 750 citations of the significant world literature on gastroenterology. The abstracts and citations are listed under three main categories: Clinical Sciences, Diagnostic Procedures, and Gastrointestinal Diseases, and these categories are subdivided into more specific groupings. Each issue contains subject and author indices that are cumulated annually.

Evaluating and editing of the abstracts and citations for relevancy and quality prior to publication is performed by the staff.

GASTROENTEROLOGY ABSTRACTS AND CITATIONS is available free upon request to qualified investigators with NIH grants and contracts in the field of gastroenterology, to other government agencies with responsibilities in this area, and to medical school libraries. In addition to the free distribution, the publication is for sale at a nominal fee by the Superintendent of Documents, U.S. Government Printing Office. The mailing list was circularized to insure currency.

Project Title:	INDEX OF DERMATOLOGY	
Professional Personnel:	Mrs. Elsie C. Yuen Mrs. Billie B. Mackey	
Man Years:	Total:	.4
	Professional:	.2
	Other:	.2

Project Description

To support the NIAMDD Dermatology Program, and respond to an increased interest and demand for up-to-date clinical studies and research information in dermatology, NIAMDD has been publishing the INDEX OF DERMATOLOGY since April 1972.

The INDEX OF DERMATOLOGY is a monthly recurring bibliography produced by the Scientific Communications Section with the aid of MEDLARS, the Medical Literature Analysis and Retrieval System of the National Library of Medicine. About 1300 dermatology-relevant citations are computer-selected each month from MEDLARS for the corresponding month, and listed under general clinical, biology, and/or cell component sections, and under an author index. The hierarchical format allows titles in a related subject area to be grouped, on the basis of their clinical or research orientation, under a specific subject heading.

The retrieval is monitored and the camera copy of the bibliography is edited prior to publication. Subject headings without retrieval are checked and noted to assure that relevant citations have not been omitted. The formulation is updated annually, in keeping with changes the controlled vocabulary undergoes every year.

To keep the bibliography useful and timely, the staff draws on the expertise of the Editorial Advisors, to insure that the INDEX's coverage is adequate and current. To evaluate the effectiveness of the INDEX as a research tool, a questionnaire survey of users was done in May 1978. From 379 mailings, 120 questionnaires were returned. The tabulated responses were generally favorable.

The INDEX OF DERMATOLOGY is available free to qualified investigators with NIH grants or contracts in the field of dermatology, to other government agencies with responsibilities in the area, and to medical school libraries. In addition to the free distribution, it is for sale by the Superintendent of Documents, U.S. Government Printing Office, at a nominal cost. The mailing list is circularized to insure currency.

Project Title: KIDNEY DISEASE AND NEPHROLOGY INDEX

Professional Personnel: Mrs. Elsie C. Yuen
Mrs. Billie B. Mackey

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description

KIDNEY DISEASE AND NEPHROLOGY INDEX, a bimonthly recurring bibliography, contains the most recent citations to journal and monograph literature on basic and clinical research in nephrology, urology, and end-stage kidney disease. The publication is produced by the Scientific Communications Section, NIAMDD with the aid of Medical Literature Analysis and Retrieval System (MEDLARS), the medical literature data base maintained by the National Library of Medicine.

The INDEX is divided into three parts, the Subject Section, Author Section, and the Appendix. The Subject Section contains approximately 2200 citations arranged hierarchically to group similar subjects together. The Author Section lists the articles only once in alphabetical sequence by the first author. The Appendix contains indexes by subject and author. Twice a year, the Appendix also carries a listing of technical reports of the Chronic Renal Disease Research Program that are deposited with the National Technical Information Service (NTIS). These reports are available from NTIS for a fee.

The staff monitors the computerized retrieval, edits the camera copy prior to publication, and annually updates the computer retrieval formulation to reflect changes in the controlled vocabulary.

The KIDNEY DISEASE AND NEPHROLOGY INDEX is available to National Institutes of Health grantees and contractors working in nephrology, urology, or on artificial kidney devices, to government agencies with responsibilities in these areas, and to medical school libraries. It is for sale by the Superintendent of Documents,U.S. Government Printing Office, for a fee. The free mailing list is circularized annually to insure currency.

Project Title:	ARTIFICIAL KIDNEY-CHRONIC UREMIA PROGRAM ELEVENTH ANNUAL CONTRACTORS' CONFERENCE PROCEEDINGS
Professional Personnel:	Mrs. Billie B. Mackey
Man Years:	Total: .4 Professional: .2 Other: .2

Project Description

THE PROCEEDINGS OF THE ELEVENTH ANNUAL CONTRACTORS' CONFERENCE held in Bethesda, Maryland, January 16-18, 1978 was published. Approximately 325 interested individuals including 175 contractors and their staff, consultants to the Artificial Kidney-Chronic Uremia Program, and Institute staff attended the Conference.

Contracts on peritoneal dialysis were presented and invited speakers gave their data at the Workshop on Peritoneal Dialysis, held in conjunction with the Eleventh Annual Contractors' Conference. This Workshop presented the current status of peritoneal dialysis technology and its use as a therapeutic method in the treatment of kidney disease in adults and children. A comparison of peritoneal dialysis with hemodialysis was presented, and continuous ambulatory peritoneal dialysis was discussed, as well as research needs and recommendations for future research. Presentations of existing contract-funded research and development projects were given by the investigators during three simultaneous sessions. The topics presented included carbohydrates, lipid and acetate metabolism, anemia, blood compatibility, biochemistry, physiology, nutrition, devices and experimental therapies. A poster session presented topics on devices, sorbents, membranes, and blood access. A plenary session included summaries of major points of the most significant findings given during the presentations.

The Proceedings were given worldwide distribution to those with interests in the field of nephrology and artificial kidneys. It is available through the National Technical Information Service for a nominal fee.

Project Title: ARTIFICIAL KIDNEY-CHRONIC UREMIA PROGRAM
TWELFTH ANNUAL CONTRACTORS' CONFERENCE
AND PROCEEDINGS

Professional Personnel: Dr. Robert J. Wineman
Dr. Fernando Villarroel
Mrs. Billie B. Mackey
Mrs. Elsie C. Yuen

Man Years:

Total:	.4
Professional:	.2
Other:	.2

Project Description

The Twelfth Annual Contractors' Conference was held in Bethesda, Maryland on January 15-17, 1979. Approximately 300 scientists from the U.S. and six foreign countries attended the conference including contractors and their staff, consultants to the Artificial Kidney-Chronic Uremia Program, interested scientists and Institute staff.

Presentations of existing contract-funded research and development projects were given by the investigators. Two Workshops were held on the first day, The Potential Of Exercise In Maintenance Dialysis Workshop and Can Diet Be Dialysis Sparing ? Workshop. Contracts on exercise training and diet therapy were presented during the workshops. Exercise training appears to alleviate complications such as hypertension and anemia, abnormalities in lipid, carbohydrate, and mineral metabolism in some dialysis patients. Diet therapy was considered both from cell metabolism and clinical aspects.

Studies comparing conventional hemodialysis with the newer approach to therapy known as hemofiltration were reported with the efficacy and costs of the two techniques compared and blood pressure response evaluated. Researchers reported on work to determine the longer term effects of continuous ambulatory peritoneal dialysis (CAPD). This treatment promises to be a simple and potentially less expensive form of therapy liberating the patient from the need to come to a dialysis center three times weekly for treatment. Studies are directed toward decreasing the rate of peritonitis, defining the ideal composition of the dialysate for this therapy, and improving the access devices through which dialysate is introduced into the abdominal cavity.

Research results were presented in the prime areas of the Artificial Kidney-Chronic Uremia Program with detailed scientific and technical information communicated among the top staff of the currently active research and development projects. A plenary session included summaries of major points of the most significant findings given during the presentations. The Proceedings will be published in the fall.

Project Title: ARTIFICIAL KIDNEY-CHRONIC UREMIA PROGRAM
CAN DIET BE DIALYSIS SPARING ? WORKSHOP

Professional Personnel: Dr. Robert J. Wineman
Dr. Gladys H. Hirschman
Mrs. Billie B. Mackey

Project Description

Approximately 250 individuals attended the CAN DIET BE DIALYSIS SPARING ? WORKSHOP held January 15, 1979 in Bethesda Maryland, in conjunction with the Twelfth Annual Contractors' Conference.

Many investigators, particulary in Europe believe that low nitrogen diets can postpone the need for dialysis therapy in patients. The extent that low nitrogen diets can be used in patients in place of dialysis depends upon maintaining good nutrition in the patient, the ability of the diet to reduce uremic toxicity to medically acceptable levels, and the ability of patients with renal failure to adhere to the diet.

This Workshop dealt with three aspects of diet as a therapy device. At the cellular level, the association of nutrients interaction with cell metabolism in chronic renal failure and during dialysis was discussed. Preliminary observations showed that to make progress in dealing with the role of nutrient components within the uremic patient at the cellular level, the simultaneous variations in multiple nutrients must be considered. Basic principles and dynamics of nutritional therapy during progressive nephron loss was discussed, and the final panel presented the present clinical applications in nutritional management of progressive renal failure.

Project Title: ARTIFICIAL KIDNEY-CHRONIC UREMIA PROGRAM
THE POTENTIAL OF EXERCISE IN MAINTENANCE
DIALYSIS WORKSHOP

Professional Personnel: Dr. Robert J. Wineman
Dr. Antonia C. Novello
Mrs. Billie B. Mackey

Project Description

Approximately 250 individuals attended THE POTENTIAL OF EXERCISE IN MAINTENANCE DIALYSIS WORKSHOP held January 15, 1979 in Bethesda, Maryland, in conjunction with the Twelfth Annual Contractors' Conference. Exercise training programs for hemodialysis patients were the focus of the Workshops. Speakers presented data on the potential benefits to be derived in designing exercise programs for dialysis patients.

Results of preliminary studies to determine if hemodialysis patients could participate safely in exercise training programs, and whether such training would improve endocrine, metabolic, and cardiovascular abnormalities and psychosocial functioning in these patients, have been positive. Exercise training appears to lessen complications such as hypertension and anemia as well as abnormalities in lipid, carbohydrate, and mineral metabolism in some dialysis patients.

Exercise training and, specifically, running were assessed as a treatment for the depression that is frequently encountered in hemodialysis patients. Although the evidence for treatment of depression is inconclusive at present, running was found to elevate the mood of hemodialysis patients.

Project Title: BEHAVIORAL AND PSYCHOSOCIAL ISSUES IN DIABETES CONFERENCE

Professional Personnel: Dr. Lester B. Salans
Mrs. Lois Lipsett
Mrs. Billie B. Mackey

Project Description

Behavioral And Psychosocial Issues In Diabetes Conference was held in Madison, Wisconsin on May 20-24, 1979. Approximately 45 individuals attended representing lay people who are either diabetics or parents of a diabetic child, diabetologists, internists, pediatricians, psychiatrists and psychologists.

The goals of this Conference were to apply behavioral knowledge and techniques to the problems of diabetes and their families; to attract new and established behavioral investigators into diabetes research; to actively encourage the interface between biomedical and behavioral researchers and clinicians; and to identify areas of most promise.

The results are to be published.

Project Title: CONFERENCE ON IMMUNE MECHANISMS IN RENAL DISEASE

Professional Personnel: Dr. Nancy B. Cummings
Dr. M.J. Scherbenske
Mrs. Billie B. Mackey

Project Description

This Conference was held in Bethesda, Maryland on October 2-4, 1978. This was the first cross-disciplinary meeting on immune mechanisms and renal diseases and was attended by 120 scientists in immunology, nephrology, pathology, endocrinology, and other related fields.

Forty-seven speakers discussed the major topics of structure-functional relationships; electrostatic forces and morphologic studies; glomerular mesangium and cells; immune mechanisms invading renal antigens; complexes; cryoglobulins and antigens; complement and non-complement mediators; major histocompatibility locus and disease including complement deficiency states; and immune mechanisms in systemic lupus erythematosus.

This state-of-the-art conference reviewed current knowledge of common interest in immunology and nephrology and looked at new areas for future research.

Project Title: CONSENSUS DEVELOPMENT CONFERENCE ON SURGICAL TREATMENT OF MORBID OBESITY

Professional Personnel: Dr. B.T. Burton
Dr. Gladys Hirschman
Mrs. Billie B. Mackey

Project Description

The Consensus Development Conference On Surgical Treatment Of Morbid Obesity was held in Bethesda, Maryland on December 4-5, 1978. Approximately 200 surgeons, internists, basic scientists, psychologists, psychiatrists, legal experts, patients, and representatives from health insurance convened to discuss the current status of surgical treatments for very severe or morbid obesity. The morbid form of severe obesity is defined as 100 pounds overweight or 200 percent or more of desirable weight as defined by the Metropolitan Life Insurance Company. Another way of defining morbid obesity would be in terms of Body Mass Index (BMI) or WT in kg/HT in meters^2, and morbid obesity would be a BMI of 47 or higher.

Among the surgical procedures that were reviewed included jejunoileal bypass, gastric bypass, gastroplasty, vagotomy, and jaw-wiring.

After risks, complications, and benefits were evaluated, consensus was reached that surgical intervention should be limited to morbidly obese patients with serious physical health or pscho-social handicaps who have been treated non-surgically, but have failed to show long-term improvement. A better understanding of different types of obesity is essential, more data concerning the incidence of massive obesity and the degree of risk associated with such severe obesity are needed, and better techniques to identify early in life, individuals destined to become morbidly obese are needed. Bypass operations should only be performed by a multi-disciplinary team of highly qualified physicians and surgeons committed to critical case selection, good management, and long-term follow-up.

The Proceedings of this Conference will be published in a journal.

Project Title: DIRECTIONS FOR RESEARCH IN OSTEOGENESIS IMPERFECTA WORKSHOP

Professional Personnel: Dr. Steven Gordon
Mrs. Billie B. Mackey

Project Description

The Directions For Research In Osteogenesis Imperfecta Workshop was held in Bethesda, Maryland on June 19, 1979.

There is a need to determine the basic defect in osteogenesis imperfecta. Work in progress in the laboratories reveals a diminished total collagen production in a severely affected child. Collagen synthetic patterns may allow classification of the brittle bone diseases on a biomedical basis, and genetic counseling would ultimately be facilitated through prenatal diagnosis.

This state-of-the-art conference on osteogenesis imperfecta evaluated the current research on abnormalities of collagen in osteogenesis imperfecta, planned research strategies concerning the inherited abnormalities of collagen in osteogenesis imperfecta and discussed related osteopenic diseases.

Project Title: FOURTH INTERNATIONAL CONFERENCE ON BONE MEASUREMENT PROCEEDINGS

Professional Personnel: Dr. G. Donald Whedon
Mrs. Billie B. Mackey

Project Description

The Fourth International Conference On Bone Measurement was held in Toronto, Canada on June 1-3, 1978. It was attended by 120 investigators, many of them from Europe. The first two in this series of meetings were held in Chicago, Illinois in 1970 and 1973, while the third was held in New Orleans, Louisiana in 1976. The previous meetings emphasized the development and use of the newly introduced technique of photon absorptiometry. This meeting differed from the previous ones in the greater representation of methods other than absorptiometry such as recent developments in bone measurement (radiokinetic, radiographic, Compton-scattering, neutron activation, CT-scanning and their new applications). The advantages and limitations of different methods in different biomedical contexts was presented.

The Proceedings was published and given worldwide distribution.

Project Title: HEMOPERFUSION FOR KIDNEY AND LIVER SUPPORTS AND DETOXIFICATION WORKSHOP

Professional Personnel: Dr. B.T. Burton
Mrs. Billie B. Mackey

Project Description

The Hemoperfusion For Kidney And Liver Supports And Detoxification Workshop was held in Haifa, Israel on August 18-19, 1979.

The topics presented and discussed included the removal of metabolites and poisons by hemoperfusion, biocompatibility: in vitro and in vivo studies with hemoperfusion columns, mathematical and kinetic models: studies of machine-man relationships, and intermittent and continuous measuring techniques and devices.

Project Title: PROCEEDINGS OF AN INTERNATIONAL NUTRITION CONFERENCE ON BEHAVIORAL EFFECTS OF ENERGY AND PROTEIN DEFICITS

Professional Personnel: Dr. Gerald F. Combs
Mrs. Marjorie Zukel
Mrs. Elsie C. Yuen
Mrs. Billie B. Mackey

Man Years:

Total:	.4
Professional:	.2
Other	.2

Project Description

This International Conference, held in December 1977 at the Pan American Health Organization in Washington, D.C., was cosponsored by the NIAMDD and the Food and Nutrition Board of the National Research Council, National Academy of Sciences as part of the NIAMDD responsibility in administering the U.S.-Japan Malnutrition Panel that is a member of the U.S.-Japan Cooperative Medical Science Program. The Conference attracted 130 participants from all over the world with speakers invited from Japan, Europe, Latin America, Africa and India as well as the United States.

The primary focus of the Conference was the effects of energy-protein malnutrition on behavior. The Conference had three goals: to consider the trends and developments in research on the behavioral effects of energy-protein malnutrition during the last decade, to provide an overview of current worldwide research on the subject, and to examine the research needs and opportunities of the future.

Specialists reviewed current worldwide research into this problem in animals and man, considered the contribution of cross-cultural investigation and comparisons reported in the last decade, and examined the priorities and opportunities for nutrition-behavior research in the future.

Publication of the proceedings for this Conference will make this knowledge readily available to behaviorable and neuroscientists, nutritionists, educators, epidemiologists, and other health professionals with interests in this problem.

Project Title:	PROCEEDINGS OF THE NATIONAL CONFERENCE ON CLINICAL TRIALS METHODOLOGY
Professional Personnel:	Dr. Harold P. Roth Mrs. Elsie C. Yuen Mrs. Billie B. Mackey
Man Years:	Total: .15 Professional: .10 Other: .05

Project Description

The National Conference on Clinical Trials Methodology was held October 3-4, 1977 at Bethesda, Maryland. Approximately 200-300 scientists from various clinical specialty areas involved in the design and conduct of clinical trials met to exchange ideas and to further explore many complex and unresolved issues inherent in the conduct of clinical trials. The conference was held as a result of the expressed need for more clinical trials as a means of ascertaining if new treatments and preventive measures are favorable and effective. It was thought that controlled trials can give long-term effects and reveal subtle aspects not immediately noticable. Improved methodology in conducting clinical trials can improve the benefit/cost ratio for clinical trials.

Over the two days of the conference the participants discussed such broad topics as when and how to stop a clinical trial, effective clinical trials investigators and adequate incentives, problems and solutions in patient recruitment, quality assurance of clinical data, ethical considerations in clinical trials,and the problem of communications between clinical trials investigators on sharing their experiences. The recommendations from the conference will serve as a useful guide for NIH planning policy and as a guide for anyone planning to conduct a clinical trial.

The conference was supported by several organizations, including from NIH,the Clinical Trials Committee of NIH, NCI, NEI, NHLBI, NIA, NIAID, NIAMDD, NICHHD, NIDR, NIMH, NINCDS and FIC and the Bureau of Biologics, Food and Drug Administration.

Proceedings of the conference were published as a supplement to the May 1979 issue of the journal CLINICAL PHARMACOLOGY AND THERAPEUTICS.

Project Title: PSORIASIS-DIALYSIS WORKSHOP

Professional Personnel: Dr. Laurence H. Miller
Mrs. Billie B. Mackey

Project Description

This Workshop was held in Bethesda, Maryland on April 23, 1979. Twenty individuals attended this important review of a new therapy for psoriasis patients, including representative researchers and clinicians from the fields of dermatology and nephrology.

Uncontrolled trials of dialytic therapy in psoriasis have shown remarkable improvement in skin lesions and well-being of psoriasis patients. The rationale for beneficial peritoneal or hemodialysis in psoriasis is the extraction of noxious exogenous or endogenous substances, or absorption of some beneficial substance from dialysis solution. A significant number of patients with psoriasis are so disabled by this disease and so intractable to all conventional therapy that this treatment may be a last resort.

Where the treatment has been used, prompt and impressive remissions of long-standing psoriasis have been reported for patients undergoing peritoneal dialysis. Though the results thus far are outstanding and much too consistent to be attributed to spontaneous remissions, virtually no information is available on the reasons for the improvements. Thus, the fact that psoriasis remissions last long past the dialysis period has led to speculation that dialysis removes some noxious substance which builds up slowly and is not effectively removed, or is removed and reabsorbed, by the kidney.

A review of the state-of-the-art of this therapy included T and B lymphocyte changes during dialysis, Vitamin A changes, protein-bound zinc and serum zinc changes during dialysis, as well as spermidine excretion after dialysis and the effects of dialysis on serum complement.

Clinical research was discussed for the best designs for blend or cross-over clinical studies, the selection of patients, and side-effects and complications to be encountered. New approaches were proposed for research and the clinical studies.

Project Title: SECOND INTERNATIONAL CONGRESS ON NUTRITION IN RENAL DISEASE

Professional Personnel: Dr. B.T. Burton
Mrs. Billie B. Mackey

Project Description

The Second International Congress On Nutrition In Renal Disease was held in Bologna, Italy on June 13-15, 1979. This meeting was a successor to the First International Congress On Nutrition In Renal Disease that was held in Wurzberg, West Germany, May 23-25, 1977.

This Congress was timely since there has been a plethora of new work and new findings in the field of nutrition in renal disease during the past two years.

The program was divided into State-of-the-Art presentations, panel sessions, and brief presentations of individual research papers in the form of free communications. There was also a poster session. Topics covered included the altered nitrogen metabolism in specific organs in renal failure which dealt with amino acid turnovers in the kidney in uremia, and the liver and the muscle in renal failure. Hypercatabolism in renal failure was discussed. Assessment of nutritional status in renal failure included discussion of the altered immune function as it relates to nutrition and renal failure and methods for assessing nutritional status. Lipid and carbohydrate metabolism in renal failure looked at the pathogenesis of atherosclerosis, treatment of hyperlipidemia, and the effect of diet, dialysis, and transplantation on hormonal changes in uremia.

Syndromes resulting from trace element deficiencies and excesses were considered as well as the clinical effects of aluminum. Vitamin D, calcium, and phosphorus effects in renal disease and renal failure were adressed.

Treatment with low nitrogen diets and the preservation of renal function with these low nitrogen diets was discussed as well as nutritional therapy in children. Conservative dietary treatment of patients in progressive renal failure as a means of postponing the day when such patients must be placed on maintenance dialysis and dietary regimens which permit minimal periods of dialytic therapy was discussed.

A proceedings will be published.

Project Title: SUMMARY OF WORKSHOP ON ARTIFICIAL DEVICES FOR THE CONTROL OF BLOOD GLUCOSE

Professional Personnel: Dr. Lester B. Salans
Mrs. Billie B. Mackey

Project Description

This Workshop was held December 1 and 2, 1977 at Bethesda, Maryland. The purpose of this Workshop was to determine the status of research in this area and to recommend future directions. Topics covered included the following: Instrumentation systems, applications of the artificial pancreas, influence of different algorithms on glucose regulation, biocompatible materials and intravenous techniques, insulin delivery systems, use of the artificial pancreas in the study of the pathophysiology of diabetes, and mathematical modeling and glucose regulation. Glucose sensors and new methods of insulin delivery were also discussed. Approximately 60 individuals participated.

A summary of the Workshop was published and distributed by the Institute.

ANNUAL REPORT SUMMARY
EPIDEMIOLOGY AND FIELD STUDIES BRANCH

The activities of the Branch include epidemiological studies of diabetes, arthritis, cholelithiasis, renal disease and osteoporosis at this time. The activities relating to diabetes and arthritis are based within the Southwestern Field Studies Section. This Section works in close collaboration with the Phoenix Clinical Research Section in which clinical and laboratory research, which stems primarily from the epidemiological studies, is carried out. The Metabolic Disease Unit based in Bethesda is presently actively involved in the epidemiology of renal disease.

Dr. Peter H. Bennett, Chief of the Branch, was honored by the U.S. Public Health Service Professional Association and delivered the Luther Terry Lecture at the Annual Meeting in June 1979. On April 26 he was elected to the Fellowship of the Royal College of Physicians of London. The Chief of the Metabolic Disease Unit, Dr. Robert Vought retired this year after many years with the Institute, and it was with sorrow we heard of his untimely death some months later. The year was also marked by the departure of two of our more senior staff physicians, Dr. Peter Savage of the Southwestern Field Studies Section, and Dr. Lynn Bennion of the Phoenix Clinical Research Section.

Southwestern Field Studies Section

Recent developments in the recognition of the heterogeneity of diabetes and the more precise classification of subjects with diabetes led to the investigation of the clinical characteristics of diabetes among younger Pima Indians, the examination of the frequency of islet antibodies in Pima Indians with recent onset diabetes and extensive studies of the HLA system among nondiabetic and diabetic Pima Indians.

Diabetes with an onset of below 15 years of age was exceedingly uncommon in the Pimas, but about 1% developed the disease between the age of 15 and 24 years. Of these subjects with an onset below 25 years of age, the course of hyperglycemia had been relatively severe with at least one episode of ketoacidosis in half of them. Nevertheless, none of these subjects could be considered truly insulin-dependent, even though they required insulin treatment, as none spontaneously developed ketosis. Furthermore, the majority were obese at onset. While true insulin-dependent diabetes was not identified it was concluded that the early age of onset of diabetes among Pimas was primarily due to the appearance of insulin-independent diabetes and that the severity of the syndrome represented phenotypic variation of the characteristic insulin-independent diabetes seen in extraordinary frequency in the older Pima.

These conclusions were strengthened as a result of the examination of the sera of recent onset Pima Indian diabetics age-sex matched controls. Islet antibodies were found in only two (4%) of the diabetics and other autoantibodies were present in 30% of both the cases and controls. Islet cell antibodies were, therefore, found in very low frequency among younger Pima Indian diabetics, in contrast with frequencies of 60% and over reported for

recent onset Caucasian insulin-dependent diabetics and indicating that the vast majority, if not all of the younger Pima Indian diabetics, are of the insulin-independent (Type II) type.

Since HLA-B8 and BW15 occur in increased frequencies in Caucasian insulin-dependent diabeticsand HLA-BW22 is more frequent in young Japanese diabetics, the frequency of HLA A, B, and DR antigens have been examined in Pima Indian diabetics and non-diabetics. HLA-B8 and BW15 were found to be virtually absent in the Pima Indian population, whereas a modest association between diabetes and the presence of HLA-A2 antigen was found. The significance of this observation is uncertain at this time, but illustrates the need for further examination of other chromosome 6 markers in diabetes, as well as the need for a continuing search for associations and linkage with the approximately other 40 gene markers which are available for this population.

The absence of HLA-B8 and BW15 in the Pima population and the low frequency of islet cell antibodies in diabetics of recent onset are all consistent with the clinical observations that true insulin-dependent diabetes mellitus is rare or unknown in this population. These observations corroborate other information, which suggests that insulin-dependent diabetes is primarily a disease of Caucasian populations in which the frequency of HLA-B8 and BW15 are quite high. Nevertheless, the possibility of finding other chromosome 6 markers which are associated with insulin-independent diabetes remains. This possibility will be examined using the other markers and by pedigree analysis. This approach is necessary to determine if insulin-dependent diabetes and non-insulin-dependent diabetes result from the same genotype, but on different genetic backgrounds. Alternatively, insulin independent diabetes may have as yet unidentified genetic determinants on other chromosomes.

The quantification of factors associated with the onset of diabetes has been continued. The incidence of diabetes varies enormously according to the degree of obesity and this relationship is also age and sex dependent. The most obese Pima appear to develop diabetes at an earlier age than the less obese. These observations strongly suggest that weight loss would have an important impact on reducing the incidence of diabetes in the Pima population and the documentation of the detailed relationships is necessary to prepare for a clinical trial concerning the question of whether or not diabetes can be prevented.

Investigation into the factors which relate to the development of complications within the diabetic continues. The levels of fasting and two hour post load plasma glucose levels which are associated with the specific complications of nephropathy and retinopathy have been defined and appear to discriminate equally between those with and without complications of diabetes. The relative merits of these glucose levels for the diagnosis of diabetes have been compared and the degree of diagnostic misclassification associated with each has been defined. The reproducibility of the levels was found to be the major difference between the two in terms of performance as a diagnostic tool, with the fasting level more reproducible.

The relationship of blood pressure to the development of retinopathy has been further evaluated. The relative risks of developing retinopathy are greater in those with systolic hypertension, and blood pressure remains an independent determinant of retinopathy, when stratified by twelve potential confounding variables. It has been postulated that the effect of blood pressure is mediated through increased retinal artery pressure. Investigation of this hypothesis using ophthalmodynonometry showed that retinal artery pressure was greater in those with diabetic retinopathy, but that the effects of increased retinal artery pressure were strongly corrolated, as expected, with systolic blood pressure which remained a better discriminator. The observation that the majority of subjects with asymmetric diabetic retinopathy were associated with higher retinal artery pressures, however, is consistent with the hypothesis that increased arterial pressure in the eye leads to the early development of retinopathy in the diabetic.

The Section has continued to investigate diabetes and its complications in other populations. In conjunction with the South Pacific Commission, an investigation of the frequency and impact of diabetes in the population of Guam was undertaken and revealed that diabetes was more frequent in the Chamorro population than in the Phillipino population of the territory, and showed that the age-specific rates of diabetes of both of these groups were greater than in the general United States population.

In conjunction with the South Pacific Commission and the Department of Epidemiology and Metabolic Medicine of the Royal Southern Memorial Hospital, Melbourne a survey was undertaken on Ilê Orea in the Loyalty Islands. The prevalence of diabetes and its relationships to obesity and other factors in the Melanesian and Polynesian populations of that island will be compared.

In collaboration with investigators from the University of Hiroshima the prevalence of diabetes and its vascular complications in Japanese migrants in Hawaii was compared to those in Hiroshima. The prevalence of diabetes in the Hawaiian Japanese was found to be twice as high as in the Japanese in Hiroshima, and while the Japanese in Hawaii were more obese, these differences did not account wholly for the greater diabetes prevalence in Hawaii. This suggests that either dietary factors or reduced physical activity in the Hawaiian Japanese had increased their prevalence of diabetes. The frequency of retinopathy, stratified for duration of diabetes, was similar in the Japanese subjects in Hawaii and Japan but ischemic heart disease was more frequent in the Hawaiian Japanese than in Hiroshima. These findings suggest that environmental factors play an important part in determining the frequency of macrovascular disease in diabetes, but provide no evidence that they do so in the development of microvascular complications.

The inter-relationships between maternal weight and glucose tolerance on birth weight has been investigated to determine the extent to which mild degrees of glucose intolerance in pregnancy are associated with increased morbidity and mortality. Both maternal weight and glucose tolerance during the third trimester of pregnancy were shown to have independent effects on birth weight. The influence of glucose intolerance was seen particularly when the two hour post load plasma glucose level exceeded 140 mg/dl, when over 50% of the infants

were large for gestational age. Such degrees of glucose intolerance were also associated with significantly increased still birth and infant mortality rates although these could also be predicted by pregestational glucose tolerance tests and correlated with the results of post-partum glucose tolerance tests. These data give some insight into the extent to which infant morbidity and mortality might be ameliorated by the recognition and treatment of glucose intolerance in pregnancy in mothers not previously known to have diabetes.

Investigations in arthrits have focused primarily in collecting further information concerning the relationship between the HLA and DR locus in rheumatoid arthritis and ankylosing spondilitis. Since DR typing has not yet been completed in the appropriate groups of cases and controls, no conclusions can be drawn at this time. These studies will continue in the hope that genetic factors related to the development of these diseases will be identified and that their recognition may lead to the identification of factors which precipitate the development of inflammatory polyarthritis in predisposed subjects.

Metabolic Disease Epidemiology Unit

With the retirement of Dr. Robert Vought investigation of the epidemiological aspects of goiter have been terminated. Dr. Jeffry Cutler has initiated a program in renal disease epidemiology, and at the present time has evaluated data on the frequency of renal disease in the United States population primarily using data gathered by the National Center for Health Statistics. These data will be used in part to identify priorities and plan new investigations in the field of renal disease epidemiology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 69000-14. EFS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Diabetes Mellitus in the Gila River Indian Community

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Bennett	Chief	EFSB, NIAMDD
Other:	W.C. Knowler	Staff Physician	SFSS, NIAMDD
	P.J. Savage	Staff Physician	SFSS, NIAMDD
	D. Pettitt	Staff Physician	SFSS, NIAMDD
	J.R. Lisse	Staff Physician	SFSS, NIAMDD
	E.J. Ballintine	Clinical Director	IRP, NEI

COOPERATING UNITS (if any) N.B. Rushforth, Case Western Reserve University; R. Kawate, Hiroshima University, Hiroshima, Japan; D. Doniach, Middlesex Hospital Medical School, London, U.K.; R.C. Williams, Arizona State University

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.2	3.5	4.7

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The natural history of the various manifestations and vascular complications of diabetes mellitus are being studied and evaluated in an Amerindian community. Various associated conditions and factors, including heredity, body weight, and blood pressure, that may have a bearing on the frequency and severity of diabetes and/or its complications are under investigation.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1. Determine the prevalence and incidence of diabetes mellitus among those five years of age and over on the Gila River Indian Reservation.

2. Evaluate factors associated with the progression from prediabetic state to subclinical diabetes mellitus and to clinical diabetes.

3. Study risk factors associated with the rate of appearance and progression of vascular complications.

4. Investigate influence of tribe, age, obesity, parity, and other factors on changes in glucose tolerance and their relationship to diabetes mellitus.

Methods Employed:

1. The following tests and examinations are repeated at two yearly intervals on all Indians five years of age or more living on the Sacaton Service Unit portion of the Gila River reservation.

   a. Clinical interview
   b. Physical examination
   c. Modified glucose tolerance test and other biochemical tests

   In addition, on those aged 15 years and over:

   d. Fundus photographs
   e. Electrocardiogram
   f. Tests for vibratory sensation threshold
   g. Soft tissue radiographs for vascular calcification

2. Some of the above tests and examinations are also being done on Indians of other tribes or living under different environmental conditions and on non-Indian subjects for comparison.

Major Findings:

1. Classification of Diabetes in Pima Indians by Studies of Islet Cell Antibodies. Pancreatic islet cell antibodies (ICA) and 12 other autoantibodies were measured at the time of diabetes in 46 Pima Indians, aged 17-47 years, and in 46 age-sex matched non-diabetic controls. ICA were found in only two diabetics, aged 20 and 25, compared with none of 46 controls. ICA were not found in sera tested from two to four years before or from two to five years after the diagnosis in the 10 subjects with ICA at diagnosis, nor in 5 other subjects aged 25 years or less at diagnosis. Neither of the subjects with ICA had other autoantibodies. At least one

type of autoantibody was found in 14 (30%) of the diabetics and in 14 controls, but none was significantly associated with diabetes. This study indicates that diabetes in the Pima Indians, even those with an onset below 25 years of age, is almost entirely of type II, in that the disease is not associated with ICA, ketoacidosis, or insulin dependence.

2. Elevated Systemic and Retinal Artery Blood Pressure are Risk Factors for Diabetic Retinopathy. Diabetic retinopathy was assessed twice in 257 diabetics and 306 non-diabetics initially free of diabetic retinopathy. Examination, separated by 6 years, included ophthalmoscopy and supine blood pressure without knowledge of the clinical status, and other studies of diabetes and its complications.

Age adjusted 6 year cumulative incidence of retinal lesions (microaneurysms, hemorrhages, or neovascularization) was 1% in non-diabetics and 31% in diabetics, indicating that most in fact represented diabetic retinopathy. Diabetic retinopathy incidence was 25% at initial systolic blood pressure <.140 mm Hg and 38% at systolic blood pressure $\geq$140 mmHg (rate ratio, RR = 1.53, $p<.025$). The effect of systolic blood pressure persisted when assessed within categories of subjects stratified by a score representing retinopathy risk due to 12 potentially confounding variables.

These findings suggested that increased retinal artery pressure might also be associated with diabetic retinopathy. To test this hypothesis, independent assessments were made of diastolic retinal retinal artery pressure with an ophthalmodynamometer, supine arm blood pressure and diabetic retinopathy (microaneurysms or hemorrhages) by direct ophrhalmoscopy in 97 diabetics of 3 through 10 years duration, aged 25 through 64 years.

Diabetic retinopathy was seen in 25% of subjects. Mean retinal artery pressure was 70 mm in those with and 65 mm in those without diabetic retinopathy ($p=0.12$). Mean systolic and diastolic blood pressure were 142/84 in those with and 130/79 in those without diabetic retinopathy ($p<.05$ for each). However, once duration and systolic blood pressure were considered, there were no additional significant effects of any other variables measured. Thus retinal artery pressure by this technique, while associated with diabetic retinopathy, was also highly associated with systolic blood pressure ($r=+.65$), which was a better discriminator for diabetic retinopathy.

Because it is measured in each eye, retinal artery pressure could be more useful in asymmetric diabetic retinopathy. In 6 of 8 subjects with asymmetric diabetic retinopathy, the more severely affected eye had the higher retinal artery pressure, consistent with the hypothesis that increased arterial pressure in the eye leads to diabetic retinopathy.

3. Environmental Effects on Diabetes Mellitus and its Vascular Complications in Japanese Migrants on the Island of Hawaii. In collaboration with the University of Hiroshima, Japan, we studied the prevalence of diabetes and its vascular complications in Japanese migrants in Hawaii, in comparison with Japanese subjects in Hiroshima. The age-sex adjusted prevalence of diabetes (2 hour post-load plasma glucose $\geq$200 mg/dl) was 12.3% in Japanese in Hawaii compared to 6.9% in Japanese in Hiroshima ($p<.01$). Although the Hawaiian sample was more obese, the distribution of body weights did not account for the greater diabetes prevalence in Hawaii. Analysis of death certificates in Hawaii suggested that diabetes has become more common in Hawaiians of Japanese descent during the last 2 decades. Comparisons of diet and activity histories supported the hypothesis that a high fat, high simple carbohydrate, low complex carbohydrate diet and/or reduced physical activity levels increase the risk of diabetes.

   There was no evidence of an environmental influence on the development of microangiopathy (retinopathy) in diabetes, as the prevalence of diabetic retinopathy (stratified for diabetes duration) was similar in Japanese subjects in Hawaii and in Japan, and it was similar to previous reports from England. On the other hand, diabetes alone did not appear to account for the greater prevalence of macroangiopathy (ischemic heart disease) in Hawaiian Japanese than in Hiroshima. Thus environmental factors, possibly including diet, appear to be involved in the development of macrovascular complications of diabetes.

4. Genetics of Diabetes Mellitus in Pima Indians. The possible associations of diabetes and several genetic markers are being investigated. Among full-blooded Pimas at least 55 years of age, 81% of diabetics had the HLA-A2 antigen, compared to 62% of non-diabetics ($p<.01$). The importance of this association in diabetes etiology is under further investigation through pedigree studies. In contrast, HLA types B8 and B15, which are associated with type I (juvenile onset type) diabetes in Caucasians, are virtually absent in Pima Indians. Possible associations and linkage with approximately 40 other gene markers are also under study.

5. Comparison of Fasting and Two-hour Post-Load Plasma Glucose Concentrations in the Diagnosis of Diabetes. The frequency distributions of both fasting and two-hour post-load plasma glucose concentrations are bimodal in Pimas aged at least 25 years. Subjects with microvascular complications of diabetes had glucose concentration predominantly in the upper components of the distributions. The degree of overlap between the lower and upper components of each distribution are similar for fasting and two-hour glucose concentrations, although the fasting was more reproducible on repeat testing, suggesting that the fasting may be preferable for diagnostic purposes. The relationships of the presence of the specific

complications of retinopathy and nephropathy to both the fasting and two-hour levels was examined. Both glucose levels provided similar discrimination between subjects who have and have not developed the specific complications of diabetes.

6. High Prevalence of Diabetes in Pima Youths. Plasma glucose levels were determined two hours after a 75 gm oral carbohydrate load in 2594 Pima Indians aged 5-24 years, approximately 80 percent of the available population. Medical records of the entire population were reviewed for a previous diagnosis of or treatment for diabetes. None of the 1718 subjects below age 15, but 7 of 876 aged 15-24 had had a previous diagnosis of diabetes. Of the 7 known diabetics, 4 had been treated with insulin and 3 with oral hypoglycemic agents. Ketoacidosis had occurred in 4. Hyperglycemia in subjects 15-24 years old was much more frequent than reported in other population groups, but in the vast majority was asymptomatic and ketosis resistant. Glucose intolerance was associated with obesity. In Pima offspring, the presence of diabetes in both parents was related to glucose tolerance in those above but not below the age of 15 years. Ketosis resistant diabetes occurs frequently in Pimas above age 15 despite no excess of insulin-ketosis-prone diabetes below 15 years of age.

7. Obesity Increases Diabetes Incidence. The age-sex adjusted incidence of diabetes mellitus was 10.1±3.2 (cases/1000 person-years ± standard error) in non-obese Pima Indians (weight/height^2 <25 Kg/m^2), 24.9±2.3 cases/1000 person-years in the moderately obese (25-35 Kg/m^2), and 56.8±6.4 cases/1000 person-years in the most obese ($\geq$35 Kg/m^2), a 5.6-fold range from least to most obese. This effect of obesity on diabetes incidence is stronger than the effect of obesity on diabetes prevalence previously observed, because the association in prevalence is diluted by the weight loss in diabetics subsequent to diagnosis. Thus the marked effect of obesity on diabetes incidence is emphasized, suggesting the importance of weight control in preventing the disease.

Significance to Biomedical Research and the Program of the Institute: Diabetes has been further characterized in the Pima Indians, the population with the worlds' highest reported diabetes incidence rate. Pima diabetics are primarily of the insulin independent type, in that the disease tends not to be associated with islet cell antibodies, ketoacidosis, or HLA types B8 or B15.

Elevated blood pressure (either systemic, as measured in the arm, or retinal artery pressure) was identified as a risk factor for diabetic retinopathy. This may help in understanding the etiology of diabetic retinopathy, for which few risk factors other than diabetes itself have been identified. The finding also has important therapeutic implications, as high blood pressure is potentially reversible by treatment, and thus the suggestion is made that some diabetic retinopathy might be preventable by blood pressure control. This should be tested by a clinical trial.

Studies of diabetes in Japanese migrants to Hawaii suggest environmental factors (such as diet and physical activity) may influence the development of diabetes and macrovascular complications, but not microvascular complications.

Preliminary genetic analyses suggest that the HLA system may be involved or linked with diabetes. With further analyses of the large bank of genetic data available on the Pimas, it is hoped that further understanding of the genetics of the disease may be attained.

Proposed Course: The long-term follow-up studies of diabetes and its vascular complications will continue. Surveillance of non-diabetic subjects becoming diabetic and of diabetics progressing with their disease gives an opportunity for further study of risk factors for diabetes and its complications. Further study of risk factors for diabetes will include studies of obesity and gene markers. Further studies of the role of blood pressure plasma glucose concentration, and drug treatment on the incidence of diabetic retinopathy and nephropathy will be undertaken.

Publications:

1. Schell, L.M., Agarwal, S.S., Blumberg, B.S., Levy, H., Bennett, P.H., Laughlin, W.S., and Martin, J.P.: Distribution of Albumin Variants Naskapi and Mexico Among Aleuts, Frobisher Bay Eskimos, and Micmac, Naskapi, Mohawk, Omaha and Apache Indians. American Journal of Physical Anthropology 49:111-117, 1978.

2. Bennett, P.H.: The Epidemiology of Diabetes Mellitus and Its Complications in the Pima Indians of Arizona. In Seng, Cheah Jin, Pin, Lim, Tambyah, John A., Shanmugartnam, S., Boon, Peter Yeo Peng, Ark, Charles Ng Sen, Foo, Kevin Hg Kok. (Eds): Proceedings of the 6th Asia and Oceania Congress of Epidemiology, Singapore, 1978, Vol. 3, pp. 480-483.

3. Bennett, P.H.: Diabetes Mellitus and Its Complications in the Pima Indians of Arizona. In Keen, H., and Pickup, J.C. (Eds): Epidemiology of Diabetes and Its Vascular Complications, Proceedings of Satellite Meeting of IXth International Diabetes Federation Meeting, Bombay, 1976, pp. 45-47.

4. Bennett, P.H.: Recommendations on the Standardization of Methods and Reporting of Tests for Diabetes and Its Microvascular Complications in Epidemiologic Studies. Diabetes Care 2:98-104, 1979.

5. Kawate, R., Yamakido, M., Nishimoto, Y., Bennett, P.H., Hamman, R.F., and Knowler, W.C.: Diabetes Mellitus and Its Vascular Complications in Japanese Migrants on the Island of Hawaii. Diabetes Care 2: 161-170, 1979.

6. Knowler, W.C., Bennett, P.H., Hamman, R.F., and Miller, M.: Diabetes Incidence and Prevalence in Pima Indians: A 19-fold Greater Incidence than in Rochester, Minnesota. American Journal of Epidemiology 108: 497-504, 1978.

7. Zimmet, P., Bennett, P.H., Endo, S., and Kuberski, T.: Diabetes, Gout and Hypertension in the Pacific Islands. Noumea, New Caledonia, South Pacific Commission, 1978, 42 pp.

8. Knowler, W.C., Bennett, P.H., Bottazzo, G.F., and Doniach, D.: Islet Cell Antibodies and Diabetes Mellitus in Pima Indians. Diabetologia (in press).

9. Bennett, P.H., and Savage, P.J.: Prognostic Factors for the Development of Diabetes Mellitus in Pima Indian Children and Young Adults. In Grave, G. (Ed.): Early Detection of Potential Diabetics: The Problems and the Promise, 1979, (in press).

10. Bennett, P.H., Knowler, W.C., Rushforth, N.B., Hamman, R.F., and Savage, P.J.: The Role of Obesity in The Development of Diabetes in the Pima Indians. In Proc. of the Vth International Meeting of Endocrinology: Diabetes and Obesity, Marseille, June, (in press).

11. Savage, P.J., Hamman, R.F., and Bennett, P.H.: Prediabetes in Pima Indians. In Camerini-Davalos, R.A. (Ed.): Treatment of Early Diabetes. New York, Plenum Press, 1979, pp. 13-19 (in press).

12. Savage, P.J., Bennett, P.H., Senter, R.G., and Miller, M.: High Prevalence of Diabetes in Young Pima Indians: Evidence of Phenotypic Variation in a Genetically Isolated Population. Diabetes (in press).

13. Rushforth, N.B., Miller, M., and Bennett, P.H.: Fasting and Two-hour Post-load Glucose Levels for the Diagnosis of Diabetes: The Relationships Between Glucose Levels and Complications of Diabetes in the Pima Indians. Diabetologia (in press).

14. Bennett, P.H.: Diabetes and Heart Disease - The Magnitude of the Problem. In Scott, R.C. (Ed.): Diabetes and the Heart (in press).

NIH Contract No. N01-AM-6-2221 Starting Date: September 30, 1978
Expiration Date: September 29, 1979

Contractor: Case Western Reserve University

Money Allocated: $86,454 Man Years Purchased: 2.2

Pathogenesis of Diabetes Mellitus in the Gila River Indian Community

Some of the development of the mathematical theory and computation and analysis involved in the above project was carried under this contract with Case Western Reserve University, Cleveland, Ohio. Methodology was further developed for grouping subjects by characteristics which are bimodally distributed, and for describing relationships between glucose tolerance and various measures of obesity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69001-10 EFS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Complications and Outcome of Diabetic and Prediabetic Pregnancies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Peter H. Bennett	Chief	EFSB	NIAMDD
	William C. Knowler	Staff Physician	SFSS	NIAMDD
	Peter J. Savage	Staff Physician	SFSS	NIAMDD
	David J. Pettitt	Staff Physician	SFSS	NIAMDD

COOPERATING UNITS (if any)
Indian Health Service
Dr. Kirk A. Aleck, Dept. of Pediatrics, University of New Mexico

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To determine the effects of diabetes mellitus and prediabetes on the complications and outcome of pregnancy in women in the Gila River Indian Community.

The characteristics of all pregnancies among residents of the Gila River Indian Community will be determined. Each pregnant woman will have a modified glucose tolerance test during the third trimester and following delivery and will be retested at approximately two yearly intervals in conjunction with the prospective study of diabetes mellitus in the same community. The characteristics of the pregnancies in women who subsequently become diabetic will be compared to those among women who were diabetic at the time of pregnancy and those who remain nondiabetic.

Rev.

Project Description:

Objectives: To document the natural history and to determine the effect of diabetic and prediabetic pregnancies in a Pima Indian group with the highest recorded prevalence of diabetes mellitus. Special emphasis will be placed on complication during pregnancy and associations with mortality and morbidity in the offspring.

Methods Employed: The course and outcome of pregnancies in all female residents of the Gila River Indian Community (Sacaton Service Unit area) are followed and recorded in a standardized manner.

All patients attending prenatal clinic at Sacaton Hospital receive a modified glucose tolerance test during the third trimester of their pregnancy. These patients receive another modified glucose tolerance test 24-48 hours post partum and a chart review and patient interview are done for additional information. They are retested at approximately 2 yearly intervals to document the development or not of diabetes. They will also be retested for each subsequent pregnancy.

Infants will be examined to determine the presence of congenital anomalies and data related to mortality and morbidity will be collected and documented. At age five years, the children will be re-examined to detect any further abnormalities which may have been inapparent at birth.

Major Findings:

1. Influence of Maternal Weight and Glucose Tolerance on Frequency of Large Babies. Large for gestational age newborns have increased morbidity and mortality. Maternal weight and glucose tolerance during pregnancy were evaluated as predictors of infant size in Pima and Papago women, who have a very high prevalence of diabetes and obesity.

A plasma glucose was drawn 2 hours after an oral carbohydrate load during the third trimester of 410 pregnancies in 345 women who subsequently delivered at term (38 to 42 weeks gestation). All women were weighed after delivery. Newborns weighing 3700 grams or more were considered large for gestational age (greater than the 90th percentile at term for University of Colorado newborns).

Per cent of Infants weighing ≥3700g at birth by Maternal Weight and third trimester glucose tolerance (N=410)

Two hour Plasma Glucose (mg/dl)	Maternal Weight(Kg)				
	-64	65-74	75-84	≥ 85	Total
-99	11	28	31	46	28
100-139	14	28	40	42	32
≥140	20	67	64	50	54
Total	13	31	39	44	32

The prevalence of large infants increased with maternal weight ($p<.001$) and with 2 hour glucose ($p<.05$) when controlled for maternal weight. There was a particularly large increase at or above a 2 hour glucose of 140 mg/dl. Predicting large births allows for better treatment of associated complications, and these data demonstrate that both maternal weight and glucose tolerance should be considered in making this prediction.

2. Maternal Glucose Tolerance and Infant Morbidity and Mortality. The offspring of women with glucose intolerance have increased rates of morbidity and mortality. Plasma glucose levels were assessed 2 hours after an oral carbohydrate load in 608 Pima and Papago women in the pregestational period, 495 women in the third trimester, and 822 women in the post-partum period. Fetal outcome, expressed as stillborns (SB), infant mortality (IM), and malformations (MA), was compared according to the 2 hour glucose results.

	Two-hour Glucose Concentration (mg/dl)					
	Pregestational		Third Trimester		Post-partum	
	<139	≥140	<139	≥140	<139	≥140
N =	608		495		822	
Ratios/1000 live births						
Still births	04	35^{xx}	02	29^{xx}	05	60^{xxx}
Infant Mortality	29	109^{xxx}	16	75^{xx}	19	54^{x}
Malformations	23	83^{xx}	16	52^{ns}	17	40^{ns}

Glucose intolerance in the pregestational period was predictive of poor pregnancy outcome. Third trimester and post-partum glucose intolerance was associated with stillbirth and infant mortality, but was not significantly associated with congenital malformations in the infants.

Significance to Biomedical Research and the Program of the Institute: In view of the high prevalence of diabetes in the Pima Indians, studies in the Gila River Community provide a unique opportunity to evaluate the outcome of both prediabetic pregnancies and pregnancies with mild carbohydrate intolerance. Retrospective studies of the course of prediabetic pregnancies have to date produced conflicting results. Although it is well established that patients with severe glucose intolerance, especially those with diabetes of long duration and significant microvascular complications, have an increased prevalence of fetal mortality and maternal morbidity, it is not certain whether treatment will improve the outcome of those pregnancies in which there is mild glucose intolerance. Limited prospective studies of the benefit of treating pregnant women with mild glucose intolerance have so far yielded conflicting results. With the adoption of more strigent criteria for the treatment of mild carbohydrate intolerance in pregnancy it is hoped that this study will be able to add to the evaluation of this problem by

monitoring the impact of these therapeutic changes on the outcome of pregnancies on the reservation.

Proposed Course: The pregnancy study will continue in conjunction with the study of the natural history of diabetes mellitus in the Gila River Indian Community. The number of pregnancies recognized to be prediabetic will increase as the mothers studied develop diabetes mellitus.

Publications:

Bennett,P.H., Webner,C., and Miller,M.: Congenital Anomalies and the Diabetic and Prediabetic Pregnancy, Elliott, Katherine, and O'Connor, Maeve (Eds): Proceedings of the Ciba Foundation Symposium 63 (new series). In Pregnancy Metabolism, Diabetes and the Fetus, London, 1979, pp. 207-225.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 69,006-09 EFS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Gila River Indian Community Autopsy Study

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Bennett, Chief	EFSB, NIAMDD
Other:	W.C. Knowler, Staff Physician	SFSS, NIAMDD
	P.J. Savage, Staff Physician	SFSS, NIAMDD
	D. Pettitt, Staff Physician	SFSS, NIAMDD
	J.R. Lisse, Staff Physician	SFSS, NIAMDD

COOPERATING UNITS (if any) C. Self, Chief, Pathology Dept., Phoenix Indian Medical Center; P. LeCompte, Clinical Professor Emeritus, Harvard Medical School; L. Klein, Professor of Orthopedic Surgery and Biochemistry; Case Western Reserve Univ.; L.Orci, Chairman, Dept. of Morphology, University of Geneva

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Section

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.3	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The postmortem characteristics of Pima Indians of the Gila River Indian Community are investigated so that findings in subjects with and without diabetes mellitus can be correlated with studies in living subjects.

Postmortem examinations are performed on members of the Gila River Indian Community whenever possible, regardless of whether the subjects have prior evidence of diabetes. Tissues are examined by light and electron microscopy and the histology related to the clinical findings in the same subjects. The histology of the pancreatic islets of Langerhans is being studied in detail to describe the changes that take place in diabetes. The distribution of cell types varies considerably between different regions of the pancreas suggesting regional variation in function.

rev. 10-75

Project Descriptions: Z01 AM 69006-09 EFS

Objectives:

1. To determine the causes of death, and associated disease processes in members of the Gila River Indian Community being followed in prospective studies on arthritis, diabetes mellitus, cholelithiasis, etc.

2. To ascertain and evaluate the histopathological changes in tissues and cells as they relate to diabetes mellitus. To assess their importance and relationship to clinical findings in members of the Gila River Indian Community upon whom autopsies are performed.

3. Evaluate factors and conditions associated with increased or decreased mortality rate in the Gila River Indians.

Major Findings: Studies of the histology of the Islets of Langerhans in human pancreases revealed a gross heterogeneity of the distribution of the diffused cell types. This implies that uniform sampling methods and sensitive quantitative analysis of cell distributions are necessary for comparative studies.

Significance to Biomedical Research and the Program of the Institute: As more subjects who have been under long term ante-mortem surveillance are autopsied, more precise clinical pathologic correlations will be available for the study of diabetes mellitus than has previously been possible.

Proposed Course: The systematic collection and cataloging of tissues will continue. As sufficient specimens become available, detailed examinations of various tissues will be made. Further studies of the kidney, including electron microscopy and biochemical quantification of the insulin-glucagon-, and pancreatic polypeptide producing cells of the Islets of Langerhans will be the major activity in the coming year.

Publications: None

NIH Contract No. NO1-AM-7-2213 Starting Date: October 1, 1978
Expiration Date: September 30, 1979

Contractor: University of Geneva, Geneva, Switzerland

Money Allocated: $24,000 Person-Years Purchased: 1

Conducting Morphological Studies of Diabetes

Under this contract, the detailed morphology of pancreases from Pima autopsy specimens is being studied. The different cell types of the Islets of Langerhans are being quantified using immunofluorescent techniques. Human fibroblasts isolated from diabetic and nondiabetic Pimas will be compared for morphologic differences.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69009-14 EFS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Natural History of Arthritis and Rheumatism on the Gila River Indian Community

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.H. Bennett, Chief	EFSB, NIAMDD
Other:	P.J. Savage, Staff Physician	SFSS, NIAMDD
	J.R. Lisse, Staff Associate	SFSS, NIAMDD

COOPERATING UNITS (if any)
Dean Mann, I, NCI

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Southwestern Field Studies Branch

INSTITUTE AND LOCATION
NIAMDD, NIH, Phoenix, Arizona 85014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.2	0.5

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The development and progression of various forms of arthritis is being investigated in an American Indian population. The patterns of joint disease will be examined to determine if distinctive patterns of degenerative joint disease can be recognized, and if so, their evolution and outcome will be followed. In addition, the prognostic significance of various factors related to rheumatoid arthritis and ankylosing spondylitis will be examined.

The study which includes clinical radiographic and serologic studies of joint disease is a part of a prospective study of the Gila River Indian Community, in which studies of diabetes mellitus and related diseases are being undertaken.

(Rev. 10-76)

Project Descriptions: Z01 AM 69009-14 EFS

Objectives:

1. Determine the progression of clinical, radiological and serological features of joint diseases affecting the Gila River Indians with particular attention to rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and gout.

2. Determine environmental and genetic factors associated with development and progression of arthritis and rheumatism.

3. To compare and contrast the clinical course of rheumatoid arthritis in sero-positive individuals with that of sero-negative arthritics.

4. Determine the predictive and prognostic significance of rheumatoid factor in relation to the clinical course of rheumatoid arthritis.

Methods Employed:

1. The following tests and examinations will be repeated on all Indians five years of age and over, living on the Sacaton Service Unit portion of the Gila River Reservation.

   a. Clinical interview
   b. Physical examination
   c. Radiographs of hands, feet, and cervical spine. Males over age 14 and females over age 44 will have a pelvis film taken
   d. Laboratory determinations: rheumatoid factor, uric acid.

2. The data from the above examinations and tests as well as demographic pedigree, environmental information, and data on other conditions and diseases will also be collected.

3. HLA and DR typing are performed on subjects in the rheumatoid arthritis, ankylosing spondylitis and appropriate controls.

Major Findings: The collection and determination of the HLA and DR antigens is in progress, analysis of these data are pending.

Significance to Biomedical Research and the Program of the Institute: Recently the recognition of association between the DR locus and inflammatory arthritis has reopened the search for genetic determinants and determinants for susceptibility to these diseases. Examination of these associations in subjects of different racial backgrounds may lead to the identification of specific determinants of these diseases.

Proposed Course: Collaborative efforts to define B lymphocyte associated antigens and ankylosing spondylitis and rheumatoid arthritis will be continued.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 69,011-11 EFSB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Experimental and Field Studies of Iodine Metabolism and Endemic Goiter

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert L. Vought	Chief, Metabolism Diseases Epidemiology Unit	EFSB NIAMDD
OTHER:	Freddie A. Brown	Chemist	EFSB NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH
Epidemiology and Field Studies Branch

SECTION
Metabolic Diseases Epidemiology Unit

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0		

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☑ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Project terminated.

3-0-0040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AM 69012-01 EFS

PERIOD COVERED

February 1, 1979 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Descriptive Epidemiology of Glomerulonephritis and Other Urinary Disorders

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Jeffrey A. Cutler Epidemiologist EFSB, NIAMDD

COOPERATING UNITS (if any)

Division of Analysis, National Center for Health Statistics; OASH, USPHS

LAB/BRANCH

Epidemiology and Field Studies Branch

SECTION

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project applies an orientation to specific renal and urinary diseases to analysis of previously collected data from death certification and health surveys. Distribution of morbidity and mortality by secular time, geography, and personal characteristics in the U.S. population is being compared to previous studies in selected groups. The main purposes of this work are (1) to examine the plausibility of various etiologic hypotheses, especially for glomerulonephritis, and (2) as an aid to planning epidemiologic studies, including future versions of NCHS health surveys and specific analytic studies.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To estimate the incidence, prevalence, and/or mortality of glomerulonephritis, proteinuria, and other urinary conditions with emphasis on causes of end-stage renal failure, and to draw inferences on the cause and course of these conditions.

Methods Employed: This work applies ad hoc methods of case selection to the various data bases and medical coding systems of NCHS. The presence or absence of consistency across data systems in the measures of occurrence is an important tool, since the opportunity to directly validate the data is lacking.

Major Findings: A marked excess of glomerulonephritis in non-whites and an apparent preponderance in non-metropolitan areas are finds leading to additional questions. An association between proteinuria and a history of hepatitis in certain groups is consistent with other evidence favoring a role for B virus in glomerulonephritis.

Significance: Glomerulonephritis is the cause of most end-stage renal failure, and its etiology is largely unknown. The report, Research Needs in Nephrology and Urology (Annual Report, 1977-78, p. 1044), emphasized research on this group of diseases, including its epidemiology and possible viral causes.

Proposed Course: The project will continue, using improved methodologic tools such as multiple cause-of-death coding, and the expanded urinary data set of the 1976-80 Health Examination Survey.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 70500-01 EFS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on Insulin's Regulation of Glucose Metabolism by Adipose Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lester B. Salans	Chief	SCMO, EFSB	NIAMDD
	Samuel W. Cushman	Associate Chief	SCMO, EFSB	NIAMDD
OTHER:	Elizabeth A.H. McGuire	Research Chemist	SCMO, EFSB	NIAMDD
	Paul J. Hissin	Staff Fellow	SCMO, EFSB	NIAMDD
	Lawrence J. Wardzala	Chemist	SCMO, EFSB	NIAMDD

COOPERATING UNITS (if any) Endocrine-Metabolism Division, Department of Medicine, Dartmouth Medical School Department of Biochemistry, Dartmouth Medical School
Diabetes Branch, NIAMDD, NIH
Laboratory of Metabolism, NIAAA, ADAMHA

LAB/BRANCH
Epidemiology and Field Studies

SECTION
Cellular Metabolism and Obesity

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.00	4.20	3.80

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Elucidation of the mechanism of insulin's stimulatory action on glucose metabolism and its perturbation in obesity and with altered dietary carbohydrate and fat content has been undertaken using the isolated rat adipose cell as a model. Mathematical modeling of the complex kinetics of insulin binding is in progress. Development of a ^{3}H-cytochalasin B binding assay for quantitating the number of functional glucose transport systems in purified plasma membranes directly demonstrates that insulin's effect on transport occurs through an increase in the number of these systems. Characterization of these transport systems and a detailed examination of the quantitative relationship between insulin binding and stimulation of transport are in progress. While insulin's primary effect occurs at the transport level, the adipose cell's lipolytic state appears to represent the primary regulator of the specific pathways for glucose carbon metabolism. The mechanisms controlling glucose carbon flux are under investigation. Obesity, as reflected in both the aging male Sprague Dawley rat and genetically obese Zucker Fatty rat, and altered dietary composition are accompanied by major alterations in the number of insulin-stimulatable glucose transport systems and the mechanisms regulating glucose carbon metabolism, but not in the mechanism of insulin action.

PHS-6040
(Rev. 10-76)

Objectives: Insulin's ability to stimulate the uptake and metabolism of glucose by such peripheral tissues as muscle and adipose tissue and to inhibit lipolysis in the latter tissue plays a major role in systemic glucose and lipid homeostasis in vivo in man and experimental animal. Insulin resistant states, exemplified by obesity and noninsulin-dependent diabetes mellitus, are accompanied, in turn, by perturbed systemic glucose and lipid homeostasis and a reduction in the capacity of the peripheral tissues to respond to insulin. Increasing ratios of dietary carbohydrate to fat, on the other hand, tend to ameliorate these insulin resistant states. The alterations in peripheral tissue function observed in vivo in these altered physiological and pathophysiological metabolic states are closely paralleled by similar alterations in adipose and muscle cell function studied in vitro. The overall objective of the Cellular Metabolism and Obesity Section is an elucidation of the mechanism of insulin action and its perturbation in obesity and diabetes and with altered dietary carbohydrate and fat content. Over the past several years, this objective has been approached through a detailed examination of the isolated adipose cell as a model system. The Section's specific objectives comprise the five major projects presently in progress: 1) mechanism of insulin action on the adipose cell, 2) characterization of the glucose transport system in the adipose cell, 3) alterations in adipose cell function in the aging rat model of obesity, 4) alterations in adipose cell function in the genetically obese Zucker Fatty rat, and 5) role of dietary carbohydrate and fat in regulating rat adipose cell function.

Progress: Insulin's complex interaction with specific binding sites in the plasma membrane of the adipose cell is accompanied by a rapid, concentration-dependent stimulation of glucose transport into, and subsequent, parallel enhancement of glucose metabolism by, this cell type. The mechanism of these actions of insulin are under investigation through 1) the development of a mathematical model of ^{125}I-insulin binding, 2) studies of the relationship between insulin binding and glucose transport activity under perturbed experimental conditions and 3) an examination of the interaction between insulin and epinephrine in regulating glucose carbon metabolism. To date, a one binding site model of insulin's interaction with the adipose cell has been formulated and rejected although some of the complexities of the kinetics of insulin association can be successfully explained by the hormone degradation portion of this model. Furthermore, a variety of experimental conditions have been found which appear to differentially affect insulin binding and insulin's ability to stimulate glucose transport, thereby providing several potential means of identifying that specific aspect of insulin-receptor interaction leading to enhanced glucose transport. Finally, some factor related to the adipose cell's lipolytic state may be more important in regulating the activities of the specific pathways for glucose carbon metabolism than insulin.

Glucose transport occurs in the adipose cell through a highly specific facilitated diffusion process. Insulin's stimulatory action occurs, in turn, through an increase in the maximum transport velocity. Characterizations of the adipose cell's transporter proteins has been initiated by developing an equilibrium ^{3}H-cytochalasin B binding assay

for quantitating the number of functional glucose transport systems in the purified plasma membranes of isolated rat adipose cells which have been preincubated in the absence or presence of insulin. Cytochalasin B rapidly and reversibly binds to adipose cell plasma membranes in a saturable fashion. A small, but highly specific, fraction of this binding is competitively inhibited by D-, but not by L-, glucose. Curve fitting analysis of cytochalasin B binding isotherms in the absence and presence of D-glucose permits identification of a single class of D-glucose inhibitable binding sites with a dissociation constant of 120 nM, a value comparable to the inhibitory constant of cytochalasin B for glucose transport in the membranes. These sites appear, therefore, to represent the glucose transporter. Insulin increases cytochalasin B binding by increasing the number of D-glucose inhibitable sites, and presumably, functional glucose transport systems, in rough proportion to insulin's stimulatory effect on transport activity in the intact cell.

Increasing adipose cell size in the aging rat model of obesity is accompanied by both a decreasing ability of insulin to stimulate glucose transport activity and a decreasing capacity of the cell to metabolize glucose. The mechanism of the former alteration has been examined by measuring the number of functional glucose transport systems with an equilibrium ^{3}H-cytochalasin B binding assay in purified plasma membranes prepared from isolated adipose cells of widely varying size which have been incubated in vitro in the absence or presence of insulin. While the number of functional glucose transport systems/mg plasma membrane protein in the basal state remains constant, the number of insulin-stimulatable systems decreases with increasing cell size in proportion to insulin's decreasing ability to stimulate transport activity in the intact cell. The mechanism of the latter alteration has been examined by estimating the activities of the specific pathways for glucose carbon metabolism in intact adipose cells of widely varying size through the use of increasing concentrations of differentially radiolabeled glucose in the presence of increasing insulin concentrations. Increasing cell size is accompanied by markedly decreasing de novo fatty acid synthesis and hexose monophosphate shunt activity and increasing endogenous fatty acid reesterification.

The relationships among equilibrium insulin binding, glucose transport activity, and glucose metabolism have been examined by well established techniques in isolated adipose cells of widely varying size prepared from geneticially obese Zucker Fatty rats and their lean controls of widely varying age and body weight. Preliminary results suggest that increasing age and body weight within the control animals are accompanied by increasing adipose cell size; increasing insulin binding; increasing basal, but probably unchanging insulin-stimulated, glucose transport activity; and increasing basal, but decreasing insulin-stimulated, total glucose utilization. Roughly similar relationships are observed with increasing age and body weight within the genetically obese animals except for insulin binding. However, at each age and body weight, cells from the obese, relative to the lean, animals are larger, and their basal and insulin-stimulated rates of glucose transport and metabolism are greater. Insulin binding, on the other hand, starts higher, but

decreases with increasing animal age to the level observed in the control cells before increasing again. In this model of obesity, therefore, net adipose cell function reflects the composite effects of multiple, not as yet specifically identified, factors comprising animal age, adipose cell size, and genetic obesity.

Decreasing ratios of dietary carbohydrate to fat in young, lean rats are accompanied by a decreasing ability of insulin to stimulate glucose transport activity in the adipose cell and a decreasing capacity of this cell to metabolize glucose. The mechanism of the former alteration has been examined by measuring the number of functional glucose transport systems with an equilibrium ^{3}H-cytochalasin B binding assay in the purified plasma membranes of basal and insulin-stimulated isolated adipose cells from rats raised on diets differing only in their ratio of carbohydrate to fat. While the number of functional glucose transport systems/mg plasma membrane protein in the basal state remains constant, the number of insulin-stimulatable systems decreases with decreasing dietary carbo-hydrate in proportion to insulin's decreasing ability to stimulate transport activity in the intact cell. The mechanism of the latter alteration has been examined by estimating the activities of the specific pathways for glucose carbon metabolism in intact adipose cells from rats raised on the same diets through the use of increasing concentrations of differentially radiolabeled glucose in the presence of increasing insulin concentrations. Decreasing dietary carbohydrate is accompanied by markedly decreasing de novo fatty acid synthesis and hexose monophosphate shunt activity.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 70501-01 EFS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Mechanism of Insulin Action on the Adipose Cell

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Elizabeth A.H. McGuire	Research Chemist	SCMO, EFSB NIAMDD
	Paul J. Hissin	Staff Fellow	SCMO, EFSB NIAMDD
OTHERS:	Samuel W. Cushman	Associate Chief	SCMO, EFSB NIAMDD
	Lawrence J. Wardzala	Chemist	SCMO, EFSB NIAMDD
	Lester B. Salans	Chief	SCMO, EFSB NIAMDD

COOPERATING UNITS (if any) Endocrine-Metabolism Division, Department of Medicine, Dartmouth Medical School
Diabetes Branch, NIAMDD, NIH
Laboratory of Metabolism, NIAAA, ADAMHA

LAB/BRANCH
Epidemiology and Field Studies

SECTION
Cellular Metabolism and Obesity

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.30	2.25	1.05

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insulin's complex interaction with specific binding sites in the plasma membrane of the adipose cell is accompanied by a rapid, concentration-dependent stimulation of glucose transport into, and glucose metabolism by, this cell. The mechanism of these actions of insulin are under investigation through 1) the development of a mathematical model of ^{125}I-insulin binding, 2) studies of the relationship between insulin binding and glucose transport activity under perturbed experimental conditions, and 3) an examination of the interaction between insulin and epinephrine in regulating glucose metabolism. To date, a one binding site model of insulin's interaction with the adipose cell has been rejected although some of the kinetics of insulin association can be successfully explained by the hormone degradation portion of this model. A variety of experimental conditions have been found which appear to differentially affect insulin binding and insulin's ability to stimulate glucose transport, thereby providing several potential means of identifying that specific aspect of insulin-receptor interaction leading to enhanced glucose transport. Finally, some factor related to the adipose cell's lipolytic state may be more important in regulating the activities of the specific pathways for glucose carbon metabolism than insulin.

Objectives: Insulin's hypoglycemic effect in man and experimental animal *in vivo* has long been recognized, as has been insulin's ability to stimulate the uptake and metabolism of glucose by such peripheral tissues as muscle and adipose tissue. Indeed, insulin's effects on peripheral tissue glucose metabolism *in vivo* are closely paralleled by similar effects on the metabolism of glucose by isolated muscle fibers and adipose cells *in vitro*. While adipose tissue and its constituent adipose cells probably do not represent a primary site of glucose utilization in overall glucose homeostasis in the intact organism, except perhaps in the young and rapidly growing animal, its availability and the homogeneity of the cells prepared therefrom render this tissue an ideal model system with which to examine the mechanism(s) of insulin action at the cellular and subcellular levels. The overall objective of the present investigation is to elucidate the mechanism through which insulin regulates glucose metabolism in the rat, and ultimately human, adipose cell. This mechanism comprises a complex interaction of insulin with specific binding sites in the adipose cell's plasma membrane, a rapid stimulation of glucose transport into the adipose cell, and a subsequent enhancement of the activity of a variety of specific pathways for glucose carbon metabolism in the adipose cell. Insulin also inhibits the stimulation of the adipose cell's lipolytic activity by epinephrine and interacts with this lipolytic hormone in regulating glucose carbon metabolism. The specific objectives for the current year have been to 1) begin the development of a mathematical model of the kinetics of ^{125}I-insulin association with, dissociation from, and degradation by, the isolated rat adipose cell, 2) begin studies of the quantitative relationship between insulin binding to, and insulin's stimulatory action on glucose transport activity in, the isolated rat adipose cell under experimental conditions where these two functions might be partially dissociated, and 3) continue an examination of the interaction between glucose entry into the isolated rat adipose cell and this cell's lipolytic activity in regulating glucose carbon metabolism under conditions where insulin is not antilipolytic. In addition, very preliminary studies have been undertaken in attempting to establish the conditions for a stimulatory effect of insulin on glucose transport in purified rat adipose cell plasma membranes, to collaboratively develop methods for examining the levels of intermediary metabolites in, and the redox state of, isolated rat adipose cells, and to adapt currently available methodology to studies of human adipose cell function.

Methods: In all experiments, isolated adipose cells were prepared by incubating the whole epididymal fat pads of male Sprague Dawley rats weighing 150-200 µg with collagenase, separating the liberated adipose cells from the stromal-vascular elements by thorough washing, and resuspending the washed cells as a stock cell suspension. Adipose cell size and concentration in the stock cell suspension were determined using an osmic acid fixation, Coulter Electronic Counter method.

In the first series of experiments, the kinetics of ^{125}I-insulin association, was then examined by incubating samples of adipose cells from

the stock cell suspension for varying periods of time up to 300 minutes at temperatures varying from 15-37°C in the presence of tracer ^{125}I-insulin and 0-17 μM (2.5 U/ml) native insulin. The dissociation of bound ^{125}I-insulin, was examined by adding additional native insulin at varying concentrations up to 17 μM (2.5 U/ml) and continuing the incubation for varying additional periods of time up to 300 minutes. The incubated cells were assayed for bound ^{125}I-insulin and the incubation medium, for degraded ^{125}I-insulin. The results of these experiments were analyzed using the SAAM27 compartmental modeling program implemented on an IBM 1108 computer at the National Bureau of Standards.

In the second series of experiments, the relationship between steady state insulin binding and insulin's concentration-dependent stimulation of glucose transport activity were then examined together in samples of adipose cells from the stock cell suspension under varying experimental conditions by well established techniques. An initial variable of interest was pH. Insulin binding at pH 6.0-9.0 was measured by incubating cells for 90 minutes at 24°C or for 30 minutes at 37°C in the presence of tracer ^{125}I-insulin and increasing concentrations of native insulin up to 17 μM (2.5 U/ml). Glucose transport activity over the same pH range was estimated by preincubating cells for 30 minutes at 37°C in the presence of 0-35 nM (0-5000 μU/ml) insulin, then measuring the time-dependent uptake of 1 mM ^{14}C-L-arabinose, a nonmetabolizable pentose transported exclusively by the glucose transport system. Analysis of the resulting binding and transport isotherms was undertaken by a curve fitting procedure.

In the third series of experiments, the interaction between glucose entry into the adipose cell and this cell's lipolytic activity in regulating the relative activities of the pathways for glucose carbon metabolism under conditions where insulin is not antilipolytic was then examined by incubating samples of adipose cells for 40 minutes at 37°C in the presence of 0.56 or 5.56 mM U-^{14}C-glucose, 0, 0.035, 0.07, or 0.7 nM (0, 5, 10, or 100 μU/ml, respectively) insulin, and 0 or 5.5 μM (1.0 μg/ml) epinephrine. $^{14}CO_2$, and ^{14}C-triglyceride glycerol and fatty acid production were measured. Some of the rats from which adipose cells were prepared were raised for 3 weeks prior to sacrifice on a diet containing a high ratio (71%:9%, by calories) of carbohydrate to fat in order to expand the overall capacity of the cells for glucose utilization.

Results and Discussion:

Tracer ^{125}I-insulin rapidly associates with the isolated rat adipose cell in a time- and temperature-dependent manner. At lower incubation temperatures, tracer binding ultimately attains a steady state level. With increasing incubation temperature, however, insulin degradation increases such that at 37°C tracer binding reaches an early maximal level, then declines. When high concentrations of native insulin are added to the cells during the association period, further tracer binding is prevented and that tracer already bound to the cells rapidly dissociates. The rate of dissociation appears to depend upon the concentration of native insulin added, the duration of the association period, and the incubation temperature. A one binding site mathematical model of this

complex process cannot satisfactorally account for the observed results although the degradation portion of this model does appear to explain the decrease in tracer association with longer incubation periods at higher temperatures. A two site mathematical model of insulin binding is now in the formulation and testing stages.

Steady state concentration-dependent insulin binding and insulin's concentration-dependent stimulation of glucose transport activity appear, in preliminary experiments, to be differentially affected by the pH at which these two activities are measured. Further detailed studies of these pH effects are in progress as are initial attempts to examine the observed binding and transport isotherms with a curve fitting technique.

Increasing epinephrine concentrations progressively enhance the rat adipose cell's lipolytic activity. Under conditions where the increase in the extracellular ratio of released fatty acids to serum albumin is minimized, such as with short incubation times in the presence of a high albumin concentration, epinephrine does not appear to stimulate either glucose transport activity or total glucose utilization. Increasing epinephrine and lipolysis are associated, however, with a marked inhibition of de novo fatty acid synthesis from glucose and stimulation of fatty acid reesterification.

At high epinephrine concentrations, insulin does not inhibit the stimulation of lipolysis. Furthermore, whether epinephrine is present or not, increasing insulin concentrations progressively stimulate glucose transport activity and total glucose utilization. However, the pattern of glucose carbon metabolism primarily reflects the lipolytic state of the cell at each and every insulin concentration tested. Further studies are in progress using differentially radiolabeled glucose in order to quantitate the activities of each of the major pathways for glucose carbon metabolism under these conditions. A collaborative effort has also been initiated towards measuring the intracellular levels of various glucose metabolites and the redox state of the cell under these same conditions in order to elucidate the mechanisms regulating glucose carbon metabolism. An attempt to extend these studies to human adipose cells is just beginning.

Publication:

Foley, J.E., S.W. Cushman, and L.B. Salans. Glucose transport in isolated rat adipocytes with measurements of L-arabinose uptake. Am. J. Physiol. 234: E112-E119, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 70502-01 EFS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Characterization of the Glucose Transport System in the Adipose Cell

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lawrence J. Wardzala	Chemist	SCMO, EFSB	NIAMDD
OTHERS:	Samuel W. Cushman	Associate Chief	SCMO, EFSB	NIAMDD
	Paul J. Hissin	Staff Fellow	SCMO, EFSB	NIAMDD
	Lester B. Salans	Chief	SCMO, EFSB	NIAMDD

COOPERATING UNITS (if any)

Department of Biochemistry, Dartmouth Medical School

LAB/BRANCH

Epidemiology and Field Studies

SECTION

Cellular Metabolism and Obesity

INSTITUTE AND LOCATION

NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.95	1.15	0.80

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Glucose transport occurs in the adipose cell through a highly specific facilitated diffusion process. Insulin, in turn, increases the maximum transport velocity. Characterization of the adipose cell's transporter proteins has been initiated by developing an equilibrium ^{3}H-cytochalasin B binding assay for quantitating the number of functional glucose transport systems in the purified plasma membranes of isolated rat cells which have been preincubated in the absence or presence of insulin. Cytochalasin B rapidly and reversibly binds to adipose cell plasma membranes in a saturable fashion. A small, but highly specific, fraction of this binding is competitively inhibited by D-, but not by L-, glucose. Curve fitting analysis of cytochalasin B binding isotherms in the absence and presence of D-glucose permits identification of a single class of D-glucose inhibitable binding sites with a dissociation constant of 120 nM, a value comparable to the inhibitory constant of cytochalasin B for glucose transport in these membranes. These sites appear, therefore, to represent the glucose transporter. Insulin increases cytochalasin B binding by increasing the number of D-glucose inhibitable sites, and presumably, functional glucose transport systems, in rough proportion to insulin's stimulatory effect on transport activity in the intact cell.

Objectives: Insulin's ability to stimulate the uptake and metabolism of glucose by such peripheral tissues as muscle and adipose tissue has long been recognized as one of the primary mechanisms through which this hormone contributes to systemic glucose homeostasis in vivo in both man and experimental animal. More recently, glucose transport activity has been implicated as the rate limiting step for glucose metabolism in the adipose, and probably muscle, cell under most physiological conditions. The entry of glucose into the adipose cell is mediated by a highly specific facilitated diffusion process. Insulin's stimulatory action on glucose uptake and metabolism in this cell occurs, in turn, primarily through an increase in the maximum transport velocity, either by increasing the mobility of preexisting transport systems which are functional in the absence of insulin, by increasing the total number of transport systems which continue to function at a constant mobility, or by some combination of the two. The overall objective of the present investigation is to characterize the adipose cell's glucose transporter proteins and elucidate the process through which their activities are modulated by insulin. The specific objectives for the current year have been to 1) complete the development of an equilibrium ^{3}H-cytochalasin B binding assay for quantitating the number of functional glucose transport systems in the purified plasma membranes of isolated rat adipose cells, and 2) examine the effects of incubating intact adipose cells in the absence or presence of insulin on ^{3}H-cytochalasin B binding.

Methods: In all experiments, isolated adipose cells were prepared by incubating the whole epididymal fat pads of male Sprague Dawley rats weighing 150-200 µg with collagenase, separating the liberated adipose cells from the stromal-vascular elements by thorough washing, and resuspending the washed cells as a stock cell suspension. Samples of adipose cells from the stock cell suspension were then incubated for 15 minutes at 37°C in the absence or presence of 7 nM (1000 µU/ml) insulin, washed in a tris, EDTA, sucrose buffer containing 0 or 7 nM insulin, and homogenized. Purified plasma membranes were collected using a well established ficoll gradient technique, washed, and suspended in phosphate buffered saline.

Equilibrium cytochalasin B binding was measured at 4°C by incubating plasma membranes in the presence of increasing concentrations of ^{3}H-cytochalasin B up to 2000 nM and unlabelled cytochalasin B at 0 or 20 µM. The membranes were separated from the incubation medium by centrifugation, and the membrane pellet and incubation medium were analyzed for bound and free ^{3}H-cytochalasin B, respectively. Free ^{3}H-cytochalasin B trapped in the membrane pellet was determined using U-^{14}C-sucrose. Glucose transport activity was measured at 20°C by incubating plasma membranes in the presence of 0.45 mM ^{14}C-D- and ^{3}H-L-glucose. The membranes were then separated from the incubation medium by a millipore filtration technique.

Glucose transport activity in additional samples of intact adipose cells from the same stock cell suspension was occasionally determined by a ^{14}C-L-arabinose uptake technique. Cells were preincubated for 15 minutes at 37°C in the absence or presence of 7 nM (1000 µU/ml) insulin,

then pulsed for varying times with 1 mM ^{14}C-L-arabinose, a nonmetabolizable pentose transported exclusively by the glucose transport system.

Results and Discussion: ^{3}H-cytochalasin B rapidly and reversibly binds to purified rat adipose cell plasma membranes at 4°C. Increasing ^{3}H-cytochalasin B concentrations are associated with increasing levels of binding until saturation of a large number of specific binding sites occurs at concentrations greater than 2000 nM. Analysis of the binding isotherms obtained in this fashion by a curve fitting procedure indicate the presence of a minimum of 3 classes of binding sites. Binding to one of these classes of sites is specifically and competitively inhibited by D-, but not by L-, glucose. This class of sites is characterized by a dissociation constant of roughly 120 nM and a capacity of roughly 3 pmoles/mg plasma membrane protein, thereby representing less than 10% of the total number of cytochalasin B binding sites.

Extraction of plasma membranes with dimethylmaleic anhydride removes 90% of the total plasma membrane protein without altering the characteristics of the D-glucose inhibitable cytochalasin B binding site. Such extracted plasma membranes have been reported to retain glucose transport activity. Furthermore, incubation of intact plasma membranes with cytochalasin E, an analogue of cytochalasin B, markedly inhibits cytochalasin B binding to all those sites which are not competitively inhibited by D-glucose without altering the characteristics of the D-glucose inhibitable site. Cytochalasin E does not inhibit glucose transport activity in either plasma membranes or intact adipose cells. Finally, cytochalasin B competitively inhibits glucose transport activity in adipose cell plasma membranes with an inhibitory constant of roughly 120 nM, a value closely corresponding to the dissociation constant for cytochalasin B binding to the D-glucose inhibitable cytochalasin B binding site. This class of binding sites is, therefore, tentatively identified as the adipose cell's glucose transport system. It's characteristics are, in turn, remarkably similar to those of the human red cell where considerably more studies have been undertaken.

A simplified assay for quantitating the number of functional glucose transport systems in purified rat adipose cell plasma membranes has been established on the basis of the above results. It comprises measurement of 6 point equilibrium ^{3}H-cytochalasin B binding isotherms in the presence of 2000 nM cytochalasin E and in the absence or presence of 500 mM D-glucose, subtraction of the latter from the former, and extrapolation of a Scatchard plot of these derived data to its intercept on the x-axis. Preincubation of intact isolated adipose cells with insulin markedly enhances cytochalasin B binding to the subsequently prepared purified plasma membranes, but only to the D-glucose inhibitable class of binding sites. This effect of insulin can be further localized to an increase in the number of sites in this class in the absence of alterations in this site's dissociation constant. Repetitive examination of insulin's stimulatory action by the simplified assay just described demonstrates a 4-8-fold increase in the number of D-glucose inhibitable cytochalasin B sites, and presumably, functional glucose transport systems, closely corresponding to the enhancement of glucose transport activity by insulin

in samples of the same intact adipose cells from which membranes were prepared.

Studies are presently in progress in order to characterize this process further. A new method is under development for measuring glucose transport activity in rat adipose cell plasma membranes. Preliminary results suggest that the turnover numbers of the functional transport systems in the basal and insulin-stimulated states are identical and similar to that of the human red cell transport system. A prelimiary experiment with human adipose cells further suggests the feasibility extending the present investigations in that direction. Additional studies are aimed at elucidating the mechanism through which the number of functional glucose transport systems increases in response to insulin.

Publications:

Wardzala, L.J., S.W. Cushman, and L.B. Salans. Mechanism of insulin action on glucose transport in the isolated rat adipose cell: enhancement of the number of functional transport systems (a Communication). J. Biol. Chem. 253: 8002-8005, 1978.

Wardzala, L.J. Identification of the glucose transport system in purified rat adipose cell plasma membranes using a cytochalasin B binding assay: effects of insulin and altered physiological states (a Ph.D. Dissertation). Hanover, NH: Dartmouth College, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM70503-01 EFS

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Alterations in Adipose Cell Function in the Aging Rat Model of Obesity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Samuel W. Cushman	Associate Chief	SCMO, EFSB NIAMDD
OTHERS:	Lawrence J. Wardzala	Chemist	SCMO, EFSB NIAMDD
	Lester B. Salans	Chief	SCMO, EFSB NIAMDD

COOPERATING UNITS (if any)

Endocrine-Metabolism Division, Department of Medicine
Dartmouth Medical School

LAB/BRANCH

Epidemiology and Field Studies

SECTION

Cellular Metabolism and Obesity

INSTITUTE AND LOCATION

NIAMDD, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.30	0.35	0.95

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Increasing adipose cell size in the aging rat model of obesity is accompanied by both a decreasing ability of insulin to stimulate glucose transport activity and a decreasing capacity of the cell to metabolize glucose. The mechanism of the former alteration has been examined by measuring the number of functional glucose transport systems with an equilibrium ^{3}H-cytochalasin B binding assay in purified plasma membranes prepared from isolated adipose cells of widely varying size which have been incubated in vitro in the absence or presence of insulin. While the number of functional glucose transport systems/mg plasma membrane protein in the basal state remains constant, the number of insulin-stimulatable systems decreases with increasing cell size in proportion to insulin's decreasing ability to stimulate transport activity in the intact cell. The mechanism of the latter alteration has been examined by estimating the activities of the specific pathways for glucose carbon metabolism in intact adipose cells of widely varying size through the use of increasing concentrations of differentially radiolabeled glucose in the presence of increasing insulin concentrations. Increasing cell size is accompanied by markedly decreasing de novo fatty acid synthesis and hexose monophosphate shunt activity and increasing endogenous fatty acid reesterification

Objectives: Increasing adipose cell size in the aging male Sprague Dawley rat model of obesity is accompanied by alterations in adipose cellular metabolic function in vitro which parallel those observed in human adipose cells in vitro in both spontaneous and experimental obesity, as well as the alterations in systemic glucose and lipid metabolism accompanying obesity in man and the aging rat. The overall objective of the present investigation is to describe these alterations in rat adipose cell function in detail and to elucidate the mechanisms responsible for these alterations. The specific objectives for the current year have been to 1) examine the role of the number of functional glucose transport systems in the decreasing ability of insulin to stimulate glucose transport in intact isolated adipose cells of increasing size, and 2) estimate the relative activities of several of the specific pathways for glucose carbon metabolism and, thereby, determine their respective contributions to the decreasing capacity of the enlarging adipose cell for glucose utilization.

Methods: In both series of experiments, isolated adipose cells of widely varying size were prepared by incubating the whole epididymal fat pads of male Sprague Dawley rats of widely varying age and body weight with collagenase, separating the liberated adipose cells from the stromal-vascular elements by thorough washing, and resuspending the washed cells as a stock cell suspension. Adipose cell size and concentration in the stock cell suspension were determined using an osmic acid fixation, Coulter Electronic Counter method.

In the first series of experiments, the number of functional glucose transport systems was then determined by measuring D-glucose inhibitable equilibrium ^{3}H-cytochalasin B binding to purified plasma membranes prepared from samples of the isolated adipose cells which were first incubated for 15 minutes in the absence or presence of 7 nM (1000 μU/ml) insulin. Scatchard analysis of 6 point cytochalasin B binding isotherms in the absence or presence of 500 mM D-glucose permits quantitation of the number of specific cytochalasin B binding sites in a class of sites which has tentatively been identified as the functional glucose transport system. Alterations in the number of these sites with increasing adipose cell size were also compared to alterations in glucose transport activity in the same preparations of cells by estimating the latter with a ^{14}C-L-arabinose uptake technique in additional samples of cells from the same stock cell suspensions.

In the second series of experiments, the relative activities of the hexose monophosphate shunt, triglyceride glycerol, triglyceride fatty acid, and TCA cycle pathways for glucose carbon metabolism were estimated by incubating samples of adipose cells for 60 minutes in the presence of 0.56 or 5.56 mM 1-^{14}C, 6-^{14}C, or U-^{14}C-glucose and 0, 0.035, 0.07, or 0.7 nM (0, 5, 10, or 100 μU/ml, respectively) insulin. $^{14}CO_2$, and ^{14}C-triglyceride glycerol and fatty acid production were then measured and the results analyzed using equations which relate the fluxes of individual glucose carbon atoms through each pathway to the relative activity of each pathway for the glucose molecule as a whole.

Results and Discussion: Increasing adipose cell size is accompanied by pro-

gressively increasing basal glucose transport activity, but little, if any, change in glucose transport activity in the presence of a maximally stimulating concentration of insulin. The fold response to insulin relative to basal activity, therefore, decreases. However, when glucose transport activity is expressed per unit cellular surface area, then that in the basal state remains unchanged with cellular enlargement while that in the presence of insulin decreases in parallel with the decreasing fold response.

When purified plasma membranes are prepared from adipose cells of increasing size following incubation in the absence or presence of insulin, yields of membrane protein are obtained in rough proportion to the surface area of the original intact cells, regardless of hormone exposure. The number of functional glucose transport systems/mg plasma membrane protein in the basal state, however, remains constant while that in the insulin-stimulated state decreases markedly. The alterations in glucose transport activity in the intact adipose cell which accompany increasing adipose cell size in the aging rat model of obesity are, therefore, closely paralleled by similar alterations in the number of functional glucose transport systems in the adipose cell's plasma membrane, in both the absence and presence of insulin, but only when the intact cell's activities are expressed per unit cellular surface area. Thus, innsulin appears to stimulate glucose transport activity in the small adipose cell, at least in part, by enhancing the number of functional glucose transport systems. The decrease in insulin's stimulatory action on glucose transport activity with adipose cellular enlargement appears, in turn, to reflect a decrease in the number of such insulin-stimulatable transport systems. Whether this decrease occurs through a reduction in the availability of stimulatable transport systems or in insulin's ability to activate all those systems available remains to be determined.

Increasing adipose cell size also appears to be accompanied by a decreasing overall capacity for glucose utilization. Indeed, in cells of intermediate size where insulin continues to elicit a substantial glucose transport response, a markedly decreased capacity of this cell for glucose metabolism appears to prevent translation of an insulin-stimulated rate of glucose entry into the cell into a stimulated rate of glucose utilization. Under these conditions, an accumulation of unmetabolized glucose can be indirectly demonstrated using an equilibrium ^{14}C-3-O-methyl-glucose countertransport technique.

This decreasing capacity for glucose utilization with adipose cellular enlargement appears to comprise markedly decreased rates of de novo fatty acid synthesis from glucose and glucose oxidation through the hexose monophosphate shunt. In contrast, the rates of α-glycerophosphate production from glucose and endogenous fatty acid reesterification, as well as glucose oxidation through the TCA cycle, are increased. The latter increases, however, do not entirely compensate for the former decreases so that overall glucose utilization declines. These alterations are apparent in both the absence and presence of insulin. Indeed, insulin's effects on the relative activities of these pathways can be entirely explained on the basis of insulin's role in determining the rate at which glucose enters cells of any size. Similar relative activities are observed at a given rate of glucose entry in cells of a given size whether that rate is achieved in the presence of insulin and a low

extracellular glucose concentration, or in the presence of a high extracellular glucose concentration alone. Furthermore, preliminary studies suggest that the lipolytic state of the cell and the availability of endogenous fatty acids for reesterification correlate more closely with the cell size-associated shifts in glucose metabolism than do any of the alterations in insulin action. The mechanisms regulating the activities of the specific pathways for glucose carbon metabolism and their perturbation with increasing adipose cell size in obesity are under further study.

Publication:

Cushman, S.W., and L.B. Salans. Determination of adipose cell size and number in suspensions of isolated rat and human adipose cells. J. Lipid Res. 19: 269-273, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 70504-01 EFS

PERIOD COVERED
October 1, 1978 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Alterations in Adipose Cell Function in the Genetically Obese Zucker Fatty Rat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Samuel W. Cushman	Associate Chief	SCMO, EFSB	NIAMDD
OTHERS:	Lester B. Salans	Chief	SCMO, EFSB	NIAMDD

COOPERATING UNITS (if any)
N/A

LAB/BRANCH
Epidemiology and Field Studies

SECTION
Cellular Metabolism and Obesity

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.50	0.10	0.40

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The relationships among equilibrium insulin binding, glucose transport activity, and glucose metabolism have been examined in isolated adipose cells of widely varying size prepared from genetically obese Zucker Fatty rats and their lean controls of widely varying age and body weight. Preliminary results suggest that increasing age and body weight within the control animals are accompanied by increasing adipose cell size; increasing insulin binding; increasing basal, but probably unchanging insulin-stimulated, glucose transport activity; and increasing basal, but decreasing insulin-stimulated, total glucose utilization. Roughly similar relationships are observed with increasing age and body weight within the genetically obese animals except for insulin binding. However, at each age and body weight, cells from the obese, relative to the lean, animals are larger, and their basal and insulin-stimulated rates of glucose transport and metabolism are greater. Insulin binding, on the other hand, starts higher, but decreases with increasing animal age to the level observed in the control cells before increasing again. In this model of obesity, therefore, net adipose cell function reflects the composite effects of multiple, not as yet specifically identified, factors comprising animal age, adipose cell size, and genetic obesity.

Objectives: Interpretation of alterations in adipose cell function with increasing adipose cell size in the aging rat model of obesity is complicated by the as yet undefined role of animal age as a potential regulatory factor. Any one of several genetic models of obesity in rodents might very well permit an examination of the relationship between altered adipose cell size and function in animals of identical age, as well as a further examination of the effects of age within groups of lean and obese animals. The overall objective of the present investigation is to describe the alterations in rat adipose cell function which accompany increasing adipose cell size in genetically obese Zucker Fatty rats and their lean controls as a function of animal age and body weight, to relate these alterations to those observed in the aging Sprague Dawley rat model of obesity, and to elucidate the mechanisms responsible for any differences between these two models. The specific objectives for the current year have been to 1) complete the initial experimental portion of the investigation entailing studies of equilibrium insulin binding, glucose transport activity, and glucose metabolism in isolated adipose cells prepared from lean and obese animals of 4, 6, 10, 15, 20, and 35 weeks of age, and 2) begin tabulation of the results of these studies in preparation for a detailed statistical analysis thereof.

Methods: Male genetically obese Zucker Fatty rats and their lean controls were obtained either at weaning or at 5 weeks of age and raised on a diet of standard laboratory chow until attaining the desired age. Animals were sacrificed and samples of blood obtained and prepared for eventual determination of plasma glucose and insulin levels. Isolated adipose cells were prepared by incubating the whole epididymal fat pads with collagenase, separating the liberated adipose cells from the stromal-vascular elements by thorough washing, and resuspending the washed cells as a stock cell suspension. Adipose cell size and concentration in the stock cell suspension were determined using an osmic acid fixation, Coulter Electronic Counter method.

Equilibrium insulin binding, glucose transport activity, and glucose metabolism were then examined in samples of adipose cells from the stock cell suspension by well established techniques. Insulin binding was measured by incubating cells for 60 minutes at 24°C in the presence of tracer ^{125}I-insulin and increasing concentrations of native insulin up to 17 μM (2.5 U/ml). Glucose transport activity was estimated by preincubating cells for 30 minutes at 37°C in the presence of 0-35 nM (0-5000 μU/ml) insulin, then measuring the time-dependent uptake of 1 mM ^{14}C-L-arabinose, a non-metabolizable pentose transported exclusively by the glucose transport system. Glucose metabolism was examined by incubating cells for 60 minutes at 37°C in the presence of 0.56-111 mM 1-^{14}C-glucose and 0-35 nM (0-5000 μU/ml) insulin, then measuring the incorporation of ^{14}C-glucose carbons into CO_2 and triglyceride glycerol and fatty acids. The sum of the incorporation into these three parameters was considered an estimate of "total" glucose utilization.

Results and Discussion: Increasing animal age in the lean control animals is accompanied by progressively increasing rat body weight and adipose cell size. That in the obese animals is accompanied by progressively increasing rat body weight, but increasing adipose cell size only up to a maximum, attained

at 10 weeks of age. At each age, however, adipose cells are much larger in the obese, than lean, rats.

Increasing rat age and body weight in the lean control animals are associated with alterations in adipose cell function which closely parallel those observed in the aging Sprague Dawley rat model of obesity. These alterations comprise 1) a progressive increase in insulin binding, 2) increasing basal, but unchanging maximally insulin-stimulated, glucose transport activity, 3) increasing basal, but decreasing maximally insulin-stimulated, "total" glucose utilization in the presence of a low glucose concentration where transport is the rate limiting step for metabolism, and 4) a markedly decreasing maximal capacity for glucose utilization. A shift in the pattern of glucose carbon metabolism to endogenous fatty acid reesterification from de novo fatty acid synthesis is also observed.

Increasing rat age and body weight in the obese animals are, in turn, accompanied by almost identical alterations in adipose cell function. However, these age-related alterations in cell function are superimposed on specific additional alterations observed in comparisons of cells from the lean and obese animals at each age and, therefore, directly attributable to an effect of obesity per se. These additional alterations comprise an enhancement in the larger cells from the obese animals relative to the much smaller cells from the lean animals of each and every parameter of cell function examined. For example, at all ages examined, both basal and maximally insulin-stimulated "total" glucose utilization are greater in cells from the obese, than lean, rats as are the maximal capacities for glucose utilization, in spite of similar alterations in these parameters in both cell types as a function of age. Insulin binding, on the other hand, appears to represent the one exception to this generalization. While insulin binding is greater to the larger, than smaller, cells at early animal ages, it decreases with increasing animal age in the larger cells until that level observed in the smaller cells is attained by approximately 15 weeks. Increasing age beyond 15 weeks is then accompanied by gradually increasing insulin binding to both cell types in parallel.

These results suggest that net adipose cell function reflects a complex interaction of multiple factors at least three of which are represented by age, adipose cell size, and some unidentified variable related to genetic obesity. Detailed analyses of the present results remain to be completed in an attempt to evaluate these complex interactions.

Publication:

Cushman, S.W., M.J. Zarnowski, A.J. Franzusoff, and L.B. Salans. Alterations in glucose metabolism and its stimulation by insulin in isolated adipose cells during the development of genetic obesity in the Zucker Fatty rat (a Preliminary Report). Metabolism 27 (Suppl. 2): 1930-1940, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AM 70505-01 EFS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Role of Dietary Carbohydrate and Fat in Regulating Rat Adipose Cell Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Samuel B. Cushman	Associate Chief	SCMO, EFSB, NIAMDD
OTHERS:	Lawrence J. Wardzala	Chemist	SCMO, EFSB, NIAMDD
	Lester B. Salans	Chief	SCMO, EFSB, NIAMDD

COOPERATING UNITS (if any)
Endocrine-Metabolism Division, Department of Medicine, Dartmouth Medical School

LAB/BRANCH
Epidemiology and Field Studies

SECTION
Cellular Metabolism and Obesity

INSTITUTE AND LOCATION
NIAMDD, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.95	0.35	0.60

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Decreasing ratios of dietary carbohydrate to fat in young, lean rats are accompanied by a decreasing ability of insulin to stimulate glucose transport activity in the adipose cell and a decreasing capacity of this cell to metabolize glucose. The mechanism of the former alteration has been examined by measuring the number of functional glucose transport systems with an equilibrium ^{3}H-cytochalasin B binding assay in the purified plasma membranes of basal and insulin-stimulated isolated adipose cells from rats raised on diets differing only in their ratio of carbohydrate to fat. While the number of functional glucose transport systems/mg plasma membrane protein in the basal state remains constant, the number of insulin-stimulatable systems decreases with decreasing dietary carbohydrate in proportion to insulin's decreasing ability to stimulate transport activity in the intact cell. The mechanism of the latter alteration has been examined by estimating the activities of the specific pathways for glucose carbon metabolism in intact adipose cells from rats raised on the same diets through the use of increasing concentrations of differentially radiolabeled glucose in the presence of increasing insulin concentrations. Decreasing dietary carbohydrate is accompanied by markedly decreasing de novo fatty acid synthesis and hexose monophosphate shunt activity.

Objectives: Variations in the ratio of dietary carbohydrate to fat in young, lean Sprague Dawly rats are accompanied by alterations in adipose cellular metabolic function in vitro which parallel those observed in human adipose cells in vitro, as well as the alterations in systemic glucose and lipid metabolism in vivo accompanying such manipulations of dietary composition in both man and rat. In addition, many of the alterations, at both the systemic and cellular levels, produced by decreasing ratios of dietary carbohydrate to fat parallel those observed with increasing adipose cell size in both spontaneous and experimental obesity in man and in the aging male Sprague Dawley rat model of obesity. Furthermore, most of the specific alterations in adipose cell function produced by increasing ratios of dietary carbohydrate to fat in the young, lean Sprague Dawley rat parallel those associated with genetic obesity in Zucker Fatty rats of the same age. The overall objective of the present investigation is to describe these dietary-induced alterations in rat adipose cell function in detail, to relate these alterations to those observed in the aging Sprague Dawley and genetically obese Zucker Fatty rat models of obesity, and to elucidate the mechanisms through which the multiple variables represented by these diverse models interact in regulating net adipose cellular metabolic activity. The specific objectives for the current year have been to 1) examine the role of the number of functional glucose transport systems in the decreasing ability of insulin to stimulate glucose transport in the intact isolated adipose cells from young, lean rats raised on diets containing a decreasing ratio of carbohydrate to fat, and 2) estimate the relative activities of several of the specific pathways for glucose carbon metabolism and, thereby, determine their respective contributions to the decreasing capacity of adipose cells from rats consuming diets of decreasing carbohydrate content for glucose utilization.

Methods: In the first series of experiments, weanling rats were raised for three weeks on one of two experimental diets of equicaloric density containing either a low (30%:50%) or high (71%:9%) ratio (by calories) of carbohydrate to fat. The protein content of the diets was maintained at a constant 20%. In the second series, rats of increasing age and body weight were obtained, then raised for an additional three weeks on either of the same two experimental diets. In both series of experiments, isolated adipose cells were prepared from these rats by incubating the whole epididymal fat pads with collagenase, separating the liberated adipose cells from the stromal-vascular elements by thorough washing, and resuspending the washed cells as a stock cell suspension. Adipose cell size and concentration in the stock cell suspension were determined using an osmic acid fixation, Coulter Electronic Counter method.

In the first series of experiments, the number of functional glucose transport systems was then determined by measuring D-glucose inhibitable equilibrium ^{3}H-cytochalasin B binding to purified plasma membranes prepared from samples of the isolated adipose cells which were first incubated for 15 minutes in the absence or presence of 7 nM (1000 μU/ml) insulin. Scatchard analysis of 6 point cytochalasin B binding isotherms in the absence or presence of 500 mM D-glucose permits quantitation of the number of specific cytochalasin B binding sites in a class of sites which has

tentatively been identified as the functional glucose transport system. Alterations in the number of these sites with altered dietary composition were also compared to alterations in glucose transport activity in the same preparations of cells by estimating the latter with a ^{14}C-L-arabinose uptake technique in additional samples of cells from the same stock cell suspensions.

In the second series of experiments, the relative activities of the hexose monophosphate shunt, triglyceride glycerol, triglyceride fatty acid, and TCA cycle pathways for glucose carbon metabolism were estimated by incubating samples of adipose cells for 60 minutes in the presence of 0.56 or 5.56 mM 1-^{14}C, 6-^{14}C, or U-^{14}C-glucose and 0, 0.035, 0.07, or 0.7 nM (0, 5, 10, or 100 μU/ml, respectively) insulin. $^{14}CO_2$, and ^{14}C-triglyceride glycerol and fatty acid production were then measured and the results analyzed using equations which relate the fluxes of individual glucose carbon atoms through each pathway to the relative activity of each pathway for the glucose molecule as a whole.

Results and Discussion: Decreasing dietary ratios of carbohydrate to fat are accompanied by unchanging glucose transport activity in isolated adipose cells in the basal state, but decreasing glucose transport activity in the presence of a maximally stimulating concentration of insulin. The fold response to insulin relative to basal activity, therefore, also decreases. These alterations occur in the absence of changes of adipose cell size and, thereby, parallel those observed with increasing cell size in the aging rat model of obesity when the latter are expressed per unit cellular surface area.

When the purified plasma membranes of basal and insulin-stimulated adipose cells from rats raised on the two experimental diets are prepared, identical yields of membrane protein are obtained, regardless of hormone exposure. However, while the number of functional glucose transport systems/mg plasma membrane protein in the basal state remains constant, that in the insulin-stimulated state decreases with decreasing ratio of dietary carbohydrate to fat. The alterations in glucose transport activity in the intact adipose cell which accompany variations in the carbohydrate and fat content of the diet are, therefore, closely paralleled by similar alterations in the number of functional glucose transport systems in the adipose cell's plasma membrane, in both the absence and presence of insulin. Thus, insulin appears to stimulate glucose transport activity, at least in part, by enhancing the number of functional glucose transport systems. The variation in insulin's stimulatory action on glucose transport activity with altered dietary composition appears, in turn, to reflect variations in the number of such insulin-stimulatable transport systems. Whether this occurs through alterations in the availability of stimulatable transport systems or in insulin's ability to activate all those systems available remains to be determined.

Decreasing ratios of dietary carbohydrate to fat also appear to be accompanied by a decreasing overall capacity of the adipose cell for glucose utilization. The magnitude of this effect of altered dietary composition is, however, progressively diminished with

increasing adipose cell size in rats of increasing age and body weight. This decreasing capacity for glucose utilization appears, in turn, to comprise markedly decreased capacities for de novo fatty acid synthesis from glucose and glucose oxidation through the hexose monophosphate shunt. These alterations are apparent in both the absence and presence of insulin. Indeed, insulin's effects on the relative activities of these pathways can be entirely explained on the basis of insulin's role in determining the rate at which glucose enters cells from rats raised on either diet. Similar relative activities are observed at a given rate of glucose entry in cells of a given size whether that rate is achieved in the presence of insulin and a low extracellular glucose concentration or in the presence of a high extracellular glucose concentration alone, and whether cells are obtained from animals raised on the low or high carbohydrate diet. Furthermore, the shift in the relative activities of these pathways which occurs with increasing adipose cell size is observed in the absence or presence of insulin and regardless of dietary composition. The mechanisms regulating the activities of the specific pathways for glucose carbon metabolism and their perturbation with increasing adipose cell size in obesity are under further study, as are the mechanisms which regulate the adipose cell's overall capacity for glucose utilization.

Publications: None

ANNUAL REPORT

OFFICE OF SCIENTIFIC AND TECHNICAL REPORTS

The Office of Scientific and Technical Reports prepares, produces and disseminates information about the varied research and program activities of the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD). This information takes many forms: brochures, pamphlets, fact sheets, background papers, announcements, news releases, reports to Congress, responses to public and Congressional inquiries, exhibits at scientific meetings, and, importantly, contact with print and broadcast media representatives.

NIAMDD extramural programs are organized into four disease areas: Arthritis, Bone and Skin Diseases Program; Diabetes, Endocrine and Metabolic Diseases Program; Digestive Diseases and Nutrition Program; Kidney, Urologic and Blood Diseases Program. Information responsibilities are divided along these lines, with an Information Specialist designated to cover the activities of each cluster. Intramural branches are assigned to the Information Specialist who covers the corresponding extramural disease area program.

Editorial assistants have been assigned to the exhibits and publications programs, respectively, in addition to their normal duties of responding to correspondence and public inquiries.

PUBLICATIONS PROGRAM

During FY 1979, OSTR was active in the preparation of new publications aimed at the lay reader. A pamphlet entitled, "Scoliosis," a first-time publication of NIAMDD, was published in response to numerous requests for current information on this orthopedic problem. A screening guide for parents was a key feature of the pamphlet. "Scoliosis" was featured by Dr. Art Uhline on the TODAY show, April 5, 1979. Over 1,000 copies of the pamphlet were distributed by the TODAY show in response to viewer requests. A new pamphlet on cystic fibrosis was prepared and widely distributed by OSTR, in anticipation of increased emphasis on cystic fibrosis research by Congress and NIH.

In the digestive diseases area, the pamphlet, "Peptic Ulcer," explains the nature and treatment for this familiar affliction. "Progress Against the Rheumatic Diseases" was published in booklet form to provide an overview of developments in the arthritis field during the period 1967-1977.

Fact sheets were published on systemic lupus erythematosus, scleroderma (progressive systemic sclerosis), obesity, and diabetes research (insulin delivery systems and islet cell transplantation).

Particularly worthwhile publications of nongovernmental organizations are purchased by OSTR to supplement existing educational materials. Recent additions include pamphlets on arthritis, ileitis and colitis, irritable bowel, inflammatory bowel disease, and liver disease.

Reprints of articles written by NIAMDD personnel on juvenile diabetes, hypoglycemia, and the surgical treatment of morbid obesity added to the store of information about NIAMDD research activities. Finally, reprints of major articles about work of NIAMDD grantees, "New Tests for Connective Tissue Diseases" and "Physicians Search for Cause of Lyme Arthritis" were obtained from NIH's Research Resources Reporter for further distribution.

Approximately 82,900 publications were distributed to the public during the period July 1978 through June 1979, including 24,000 copies of "How to Cope With Arthritis." This publication was distributed through placement in booklet racks in Connecticut, Ohio and California metropolitan area grocery stores.

In addition, about 5,000 copies of the directory of Multipurpose Arthritis Centers and 3,000 copies of "Foot Care for the Diabetic Patient" were distributed upon request.

CONGRESSIONAL INQUIRIES

The Office of Scientific and Technical Reports handles constituent correspondence referred from the Congress and the White House. Because of active public interest in a wide-ranging number of subject areas within its research area, NIAMDD receives a large volume of these requests.

During the period July 1978 through June 1979, OSTR prepared 142 written responses to inquiries from Senators and Congressmen, the Secretary, DHEW, and the White House. Telephone inquiries from Congressional staff members are also directed to OSTR on a regular basis. A total of 192 such calls on a variety of subjects were handled by staff during the past year.

Most Congressional inquiries concern the funding of individual grants and specific research programs; lobbying efforts of numerous voluntary health agencies; proposed "cures" for various ailments; the Federal funding status of a particular research area; requests from constituents for financial assistance; and requests for information from ill constituents, their families and friends.

Overall, written responses to Congress and the White House during 1978-1979 increased by 36, an average of three inquiries per month over the previous year. This steady increase is expected to continue in the future, due in part to the increasing interest of citizens in the Institute's programs.

OSTR staff members prepared comprehensive special reports on five separate research areas in preparation for Congressional appropriation hearings. The reports concerned research advances in arthritis, diabetes, cystic fibrosis, digestive diseases and nutrition, and kidney disease. These reports were later published as a series and distributed to relevant publics.

In addition, OSTR prepared the budget narrative and the Director's opening statement to Congress, highlighting selected research findings of Institute scientists and grantees.

For the second year, OSTR prepared the comprehensive and Congressionally-mandated Report of the Director, NIAMDD, on the Arthritis Program. Descriptive of program initiatives and research advances, the report was published and distributed to the arthritis community and relevant Institute publics.

PUBLIC INQUIRIES

A significant portion of the U.S. public is directly concerned with the disease areas under investigation by NIAMDD. An estimated 60 million people in the U.S. are victims of arthritis, diabetes and digestive diseases alone. During the period covered by this report, OSTR received approximately 3,840 telephone inquiries from patients, their relatives and friends, students, allied health professionals, physicians and scientists. In addition, 472 letters were written in response to public inquiries, exclusive of requests for publications, or Congressional inquiries.

Requests for information dealt with questions about controversial forms of therapy, how to become a patient in an NIAMDD research study, financial aid, physician referral, accepted diagnosis and treatment procedures, proposed "cures" for certain diseases, and the NIAMDD research grant and contract program.

PRESS ACTIVITIES

Contact with representatives of print media is an integral part of OSTR's public information program. In support of the established policy to publicize NIAMDD's programs and activities, OSTR reaches a variety of media outlets through personal contact and distribution of news releases and announcements.

Highlights of media coverage in the arthritis, bone and skin diseases area included feature articles in nationally-circulated magazines on systemic lupus erythematosus, Multipurpose Arthritis Centers, and the mistreatment of arthritis. National media attention was also given to NIAMDD grantees working in the areas of electromagnetically-stimulated bone growth, Lyme arthritis, PUVA as a treatment for psoriasis, retinoic acid as treatment for acne, immune interferon and autoimmune disease, and Paget's disease.

"New Hope For Diabetics," a major feature article, appeared in Woman's Day magazine, April 24, 1979. Support for additional diabetes research was the subject of an editorial in the New York Times, June 1, 1979. National media coverage was given to the work of NIAMDD scientists and grantees in juvenile diabetes, Coxsackie virus as a cause of juvenile diabetes, the insulin pump, pancreatic islet cell transplantation, the artificial pancreas, the Southwestern Field Studies Branch study of the Pima Indians, stress and diabetes, and the overdiagnosis of diabetes.

The Digestive Diseases Program received major media coverage following a press conference in January 1979 that announced the recommendations of

the National Commission on Digestive Diseases. Editorial endorsement for the Commission report came from the Washington Post, and other media including Hospital Practice magazine, which ran an extensive analysis of Commission activities.

NIAMDD's Concensus Conference, "The Surgical Treatment of Morbid Obesity," drew national media attention by providing a forum for the discussion of the ethics, risks and benefits of this relatively new procedure.

Media coverage in the kidney disease program focused on development of continuous ambulatory peritoneal dialysis (CAPD), and kidney transplants. NIAMDD also sponsored a conference on Immune Mechanisms in Renal Disease, held on October 2-4, 1978.

OSTR issued a number of press releases during the past year, including announcements on the award of grants for Multipurpose Arthritis Centers, Diabetes Research and Training Centers, the initiation of clearinghouses in both the arthritis and diabetes programs, research progress in insulin delivery systems and islet cell transplantation, a state of the art study of cystic fibrosis, and insulin receptor disorders.

Articles on NIAMDD research appeared in the prestigious "From the NIH" column in the Journal of the American Medical Association. Topics included the traveling artificial kidney ("suitcase kidney"), synovectomy in the treatment of rheumatoid arthritis, the improved outlook for treatment of systemic lupus erythematosus, and the etiology of insulin dependent (juvenile) diabetes.

Two by-lined feature articles by Dr. G. Donald Whedon, "What You Should Know About Arthritis" and "What You Should Know About Peptic Ulcer," appeared in the nationally-circulated Pharmacy Times.

NIAMDD participated in a press conference held in San Francisco on March 23, 1979 to announce an NIAMDD grant for the Rosalind Russell Medical Research Center for Arthritis, part of the Multipurpose Arthritis Center at the University of California, San Francisco. A press conference was held by Representative Parren J. Mitchell on April 2, 1979 to announce the NIAMDD award of a $2 million, four-year grant to a consortium of universities for the study of various aspects of the skin disease, vitiligo.

NIAMDD intramural research activities involving lymphopheresis for the treatment of rheumatoid arthritis, and the U.S.-U.S.S.R. Cooperative Study in Arthritis were subjects of press interviews at the American Rheumatology Association meeting May 30-June 1, 1979.

A primary method of communication is through the NIH Record, which is distributed to every NIH employee and to wire services, local news media, science writers and editors, and scientists in the U.S. and abroad.

NIAMDD news releases, described earlier, are published by the NIH Record. In addition, during the past year articles were written for the Record on the following subjects: the Conference on Immune Mechanisms in Renal Disease; Dr. Lawrence E. Shulman's election as president of the Pan American League

Against Rheumatism; NIAMDD grantees' production of rat growth hormone; publication of the 2nd Annual Report of the Director, NIAMDD, on the Arthritis Program; Dr. Pierre De Meyts' receipt of the Prix des Alumni for his research in insulin receptors; Dr. Alfred D. Steinberg's 1978 Award in Biological Sciences from the Washington Academy of Sciences; Dr. Carl M. Leventhal's promotion to rank of Assistant Surgeon General, PHS; the appointment of Clifford Moss as Personnel Officer for NIAMDD; NIAMDD's observation of the 28th Annual Secretaries Week; the Juvenile Diabetes Award presentation to Dr. Lester B. Salans; the election of Drs. Hagins and Ashwell to the National Academy of Sciences; the death of Dr. Kehl Markley, NIAMDD burn expert; the selection of Dr. Elvin A. Kabat to deliver the R.E. Dyer Lecture on March 14, 1979; the retirement of Alice Staats, NIAMDD intramural senior secretary; and NIAMDD's sponsorship of Dr. Robert T. Shimke as the speaker for the NIH Lecture Series.

The Portfolio of Skin Diseases, a collection of photographs of NIAMDD grantees at work, was sent to medical and science writers throughout the U.S. and abroad.

RADIO AND TELEVISION

In connection with the activities of the National Commission on Digestive Diseases, Dr. Harold Roth, Associate Director, Digestive Diseases and Nutrition Program, was a frequent radio and television spokesman. Drs. Sarah C. Kalser and George Kitzes of the Digestive Diseases and Nutrition Program were interviewed on the ABC network station in Las Vegas, Nev. during Digestive Diseases Week. Dr. Laurence H. Miller, Skin Diseases Program Director, was interviewed on the local Channel 9 Panorama show, while Dr. Paul di Sant'Agnese was interviewed on Channel 9 news about cystic fibrosis research.

NIAMDD Associate Directors and program directors were interviewed for radio broadcast on a variety of research subjects over the past year. Taped interviews that were aired locally and distributed to radio stations throughout the country featured Dr. Harold Roth, Dr. Lester Salans, Dr. John Decker, Dr. Denis McCarthy, Dr. Gerald Combs, and Dr. Laurence Miller.

EXHIBITS

OSTR has responsibility for coordinating the exhibit program for NIAMDD, designed primarily for a scientific audience. Five major exhibits were viewed at various medical and scientific meetings throughout the U.S. by approximately 60,000 physicians, scientists and allied health professionals.

Institute exhibits on artificial kidney research, human growth hormone, cystic fibrosis and general research were displayed at meetings such as those of the National Kidney Foundation, American College of Physicians, Federation of American Societies for Experimental Biology, American Society for Artificial Internal Organs, the American Society of Nephrology, American Gastroenterology Association, American Diabetes Association, New York State Medical Society and the College of American Pathologists. Appropriate publications were distributed by OSTR staff members who accompanied exhibits.

During the past year, the exhibits program has been supplemented by the use of portable displays. Individual panels that are easily updated have been designed for the human growth hormone program, the overall NIAMDD research program, and the diabetes extramural program.

RESEARCH REPORTS

Research reports are brief summaries about significant findings reported in the literature by NIAMDD scientists and grantees. Prepared by OSTR, these reports reflect the entire scope of the Institute's research programs, and are used primarily as a basis for the special reports to Congress and related reports to DHEW and NIH.

The following examples are representative of the approximately 35 research reports prepared during the past year: 1) specific hemoglobin gene sequences have been identified, affirming the possibility of prenatal diagnosis of thalassemia, an inherited hemolytic anemia; 2) a follow-up of adults with maturity-onset diabetes showed no evidence that insulin prevents or reduces mortality from vascular complications; 3) hypertension following kidney transplants depends upon excess angiotensin II in patients with more than one kidney; 4) a distinct syndrome that is characterized by excess urinary uric acid and kidney stone disease is successfully treated with allopurinol; 5) all infant formulas, other than the meat-based variety, supply less selenium (an essential trace element) per day than breast milk, and thus should be supplemented for long-term use; 6) dexamethasone (a synthetic anti-inflammatory adrenal cortex hormone) response may be useful in prenatal diagnosis of cystic fibrosis; 7) a three-year experience with short daily peritoneal dialysis self-treatment showed that it may be superior as home treatment to hemodialysis performed three times per week; 8) native human Types I and II collagens may act as autoantigens, triggering tissue inflammation in rheumatoid arthritis; 9) an increase in the fiber content of the diabetic diet significantly reduces plasma glucose in insulin-dependent patients, and may represent an effective means of improving control of certain diabetics; 10) defective fat-induced inhibition of gastrin release may contribute to the increased gastric acid secretion in duodenal ulcer.

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